

10/525126

A Genomic Approach to Identification of Novel Broad-spectrum
Antimicrobial Peptides From Bony Fish

5 **BACKGROUND OF THE INVENTION**

Antimicrobial peptides have been isolated from a wide variety of plants and animals, and play an important role in defense against microbial invasion. They fall into three main classes based on secondary structure and amino acid sequence similarities: α -helical structures, highly disulphide-bonded (cysteine-rich) β -sheets and those with a high percentage of single amino acids such as proline or arginine. Most molecules are amphiphilic and contain both cationic and hydrophobic surfaces, enabling them to insert into biological membranes. Although one of the modes of action of antimicrobial peptides has been described as lysis of pathogens, they may also exert their effects by binding to intracellular targets. They have also been reported to exert a number of effects such as mediating inflammation and modulating the immune response.

A small number of natural antimicrobial peptides have been isolated from teleosts including the pleurocidin, from the skin of winter flounder (Cole, Weis et al. 1997), pardaxin from Red Sea Moses sole (Oren and Shai 1996), misgurnin from loach (Park, Lee et al. 1997), HFA-1 from hagfish (Hwang, Seo et al. 1999), piscidins from hybrid striped bass eosinophilic granule cells (Silphaduang and Noga 2001), moronecidins from hybrid striped bass (Lauth, Shike et al. 2002), parasin, a cleavage product of histone 2A from catfish (Park, Park et al. 1998) and some uncharacterized mucous secretions from carp (LeMaitre, Orange et al. 1996) and trout (Smith, Fernandes et al. 2000). In addition, a cationic steroidal antibiotic, squalamine, has been isolated from the shark, *Squalus acanthias* (Moore, Wehrli et al. 1993).

Cysteine-rich antimicrobial peptides of the defensin family have been detected in the fat body of insects and the hemolymph of molluscs and crustaceans. They have also been isolated from various epithelia of mammals as well as circulating cells such as neutrophils and macrophages. Recently, small cysteine-rich peptides exhibiting

antimicrobial activity against various fungi, Gram positive and Gram negative bacteria have been isolated from blood ultrafiltrate (Krause, Neitz et al. 2000), the human urinary tract (Park, Valore et al. 2001), and the gill of bacterially challenged hybrid striped bass (Shike et al. 2002). These peptides, referred to as hepcidin or LEAP-1 (liver-expressed antimicrobial peptide), have been proposed to be the vertebrate counterpart of insect peptides induced in the fat body in response to infection (Park, Valore et al. 2001).

Antimicrobial peptides have a variety of potential uses. (see for example US 6,288,212 of Hancock)

The conventional approach to identifying antimicrobial peptides involves biochemical purification from tissues or secretions. Fractions are tested for antimicrobial activity, and the purified peptides that exhibit activity are then sequenced. This approach is costly, time consuming, and not well suited to the identification of low abundance or difficult-to-purify antimicrobial peptides.

Thus, it is an object of the invention to provide a method for identifying potential antimicrobial peptides.

SUMMARY OF THE INVENTION

In one aspect of the invention there is provided a method of identifying candidate nucleic acid sequences encoding antimicrobial peptides, said method comprising:

- (a) identifying an initial peptide of interest;
- (b) identifying genomic DNA encoding the initial peptide;
- (c) identifying a flanking sequence on each side of the initial peptide;
- (d) obtaining primers complementary to the flanking sequences; and,
- (e) screening a wide range of nucleic acid sequences to identify candidate sequences capable of being amplified using the primers from step d).

According to one aspect of the invention the nucleotide and deduced amino acid sequences of hepcidin-like peptides are provided.

According to another aspect of the invention, the nucleotide and deduced amino acid sequences of pleurocidin – like peptides are provided.

According to another aspect of the invention primers suitable for use in the
5 identification, isolation and/or amplification of nucleic acid sequences encoding novel microbial peptides are provided.

According to another aspect of the invention there is provided a method for the identification of families of nucleic acid sequences encoding antimicrobial peptides.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Figure.1 is a textual and graphical depiction of pleurocidin WF2 cDNA from winter flounder (A), a graphical depiction of a predicted hydrophobicity plot of peptide WF2 (B), and a diagrammatic depiction of a predicted helical structure
15 of WF2 (C).

Figure 2 is a pictorial depiction of results of amplification of certain hepcidin-like cDNAs.

Figure 3 is a depiction of certain aligned pleurocidin –like peptide sequences.

Figure 4 is a pictorial depiction of the results of PCR amplification of certain
20 pleurocidin-like genomic sequences.

Figure 5 is a depiction of an extended genomic sequence of WF4.

Figure 6 is a depiction of an alignment of certain pleurocidin-like polypeptide sequences.

Figure 7 is a pictorial depiction of the results of expression of certain pleurocidin-like
25 genes in different winter flounder tissues.

Figure 8 is a pictorial depiction of the results of RTPCR of expression of certain pleurocidins during winter flounder development.

Figure 9 is a pictorial depiction of the results of a study of the expression of certain pleurocidin-like genes during winter flounder development.

Figure 10 is a pictorial depiction of the results of a Southern analysis of certain pleurocidin genes of winter flounder.

Figure 11 is a schematic depiction of the genomic organization of certain pleurocidin genes from winter flounder.

5 *Figure 12* is a schematic depiction of certain transcription factor binding sites located upstream from pleurocidin genes from winter flounder.

Figure 13 is a graphical depiction of results showing the impact of peptide NRC-15 on bacterial survival.

10 *Figure 14* is a graphical depiction of results showing the impact of peptide NRC-13 on bacterial survival.

Figure 15 is a graphical depiction of results showing the impact of peptide NRC-12 on yeast survival.

15 *Figure 16* is a depiction of nucleotide sequences of an unspliced (A) and partially spliced (B) cDNA encoding a type I hepcidin and a schematic depiction of intron/exon structure of a hepcidin gene in human, mouse and salmon (C).

Figure 17 is a depiction of certain hepcidin sequences from different species shown in alignment.

20 *Figure 18* is a depiction of certain aligned 3' untranslated regions of hepcidin genes from winter flounder (A) and Atlantic salmon (B).

Figure 19 is a pictorial depiction of the results of Southern hybridization analysis of certain hepcidins from different fish species.

Figure 20 is a pictorial depiction of the results of an assay of the expression of certain hepcidin and actin genes in various tissues of winter flounder.

25 *Figure 21* is a pictorial depiction of the results of an assay of the expression of certain Type I (A) and Type 2 (B) hepcidin and actin genes in various tissues of control and infected salmon.

Figure 22 is a pictorial depiction of the results of an assay of expression of certain Type I (A), Type II (B) and Type III (C) hepcidin and actin genes in developing winter flounder larvae.

5 *Figure 23* is a schematic depiction of steps taken in an embodiment of the method for identifying pleurocidins.

Figure 24 is a schematic depiction of steps taken in an embodiment of the method for identifying hepcidins.

Figure 25 is a graphical depiction of experimental results using antimicrobial peptide NRC-13 in the presence of 150 mM NaCl.

10 **DETAILED DESCRIPTION OF THE INVENTION**

The method of the invention builds on the surprising discovery that the flanking sequences around antimicrobial peptides, including without limitation pleurocidins and hepcidins, are conserved. The method of the invention provides a means of identifying nucleotide sequences encoding pleurocidins and hepcidins, and identifying
15 the encoded polypeptide sequences.

In one embodiment, the method provides, generally, a way of identifying members of a family of antimicrobial peptides once a single family member has been identified. The initial family member may be an initial peptide of interest. Initial peptides of
20 interest can be identified based on either known or reported antimicrobial activity or based on sequence similarity to other known antimicrobial peptides. Once an initial peptide has been identified, the genomic DNA encoding it is identified and its flanking sequences are determined.

25 As used herein, the term "flanking sequences" refers to nucleic acid sequences appearing at or near one or both ends of a target nucleic acid sequence encoding an antimicrobial peptide.

As used herein a nucleic acid sequence is "at or near" the end of a target
30 sequence if a portion of the sequence is within 50 nucleic acids of the end of the gene (whether within the coding region or outside it).

When an initial peptide of interest is identified based on sequence similarity to another peptide with known antimicrobial activity, the initial peptide preferably has an amphipathic structure and a net charge. In some instances the charge will preferably be a net positive charge of at least 2. In some instances, the peptide is at least 75 %, 85% or 95 % identical in sequence to the peptide having known antimicrobial activity. In some instances the sequence similarity identified may relate to similarity between nucleic acid sequences encoding the known peptide and encoding the peptide of interest. In such instances, the predicted peptide for the peptide of interest will be considered with respect to predicted charge and amphipathic structure.

For example, the prepro-sequences of pleurocidins and hepcidins tend to be conserved. Thus, by employing nucleic acid primers specific for such sequences, one can identify potential pleurocidin- and hepcidin- encoding sequences. Alternatively or additionally, known gene sequences of other classes of antimicrobial peptides can be examined to identify regions which appear to encode conserved prepro-sequences and a similar strategy used to identify other members of this family of peptides. The corresponding antimicrobial peptide encoded by such sequences can be predicted using the general features found in most pleurocidins and hepcidins, such as, for example, a net positive charge of at least 2 and an amphipathic structure.

As used herein with respect to pre-, pro- and prepro sequences of antimicrobial peptides, "pre" and "pro" have the following meaning: "Pre" refers to the signal peptide portion (or a functional portion thereof) of the peptide. "Pro" refers to the propiece. In pleurocidins the propiece is the anionic region at the carboxy terminus. In hepcidins the propiece is the region upstream of the mature peptide. In the non-limiting examples disclosed herein pleurocidin primers were designed based on the pre and pro regions, and hepcidin primers were designed based on the pre region and the 3' untranslated region (UTR).

PCR can be used to amplify nucleic acid sequences encoding potential pleurocidins or hepcidins. This can be conveniently accomplished by using a pair of PCR primers, one of which recognises a nucleic acid sequence complementary to a polynucleotide sequence encoding an amino-terminal prepro-sequence conserved in

the peptide type of interest, and the other complementary to a 3' conserved region in the nucleotide encoding the peptide-type of interest. It will be appreciated that other prepro-sequences may exist and are specifically contemplated. For example, redundancy in the genetic code allows for multiple nucleic acid sequences encoding a particular amino acid sequence. As discussed with respect to 5' prepro-sequences, other 3' conserved sequences may exist and are specifically contemplated. When designing primers it is useful to have reference to known codon usage information for the species in which sequence amplification is sought.

10 In an embodiment of the invention there is provided the use of signal sequence I or a nucleic acid sequence encoding same in identifying or amplifying potential pleurocidins.

Signal Sequence I

15 MKFTATFL (X)_n (L)_o (F)_p I (F)_q (X)_y VLM (X)_z (V)_r (E)_s (D)_t (P)_u (L)_v G E (C)_w (G)_x

Wherein:

n is 1 to 3	u is 0 or 1
o is 0 to 2	v is 0 or 1
p is 0 or 1	w is 0 or 1
20 r is 0 or 1	
s is 0 or 1	x is 0 or 1
t is 0 or 1	y is 0 or 1
	z is 0 or 1

with the restriction that:

25 x + o + p = 3,	s + t = 1,
u + v = 1,	w + x = 1, and
q + = 1.	

30 In an an embodiment of the invention there is provided the use of one or both sequence PL1 or PL2 or a nucleic acid sequence encoding same in identifying or amplifying potential pleurocidins.

PL1 GCCCACTTTGTATTCGCAAG

PL2 CTGAAGGCTCCTTCAAGGCG

In an embodiment of the invention there is provided the use of an acidic sequence I or a nucleic acid sequence encoding same in identifying or amplifying potential pleurocidins.

Acidic Sequence I

$(Y)_a (X)_b (X)_c (E)_d (X)_e (Q)_f (E)_g L (N/D) KR (A/S) V D (D/E)$

wherein:

10

a is 0 or 1	e is 1 to 3
b is 0 or 1	f is 0 or 1
c is 1 or 2	g is 0 or 1
d is 0 or 1	

15

with the restriction that

a + b = 1,
c + d = 2, and
e + f + g = 3.

20

As used in the sequences herein "X" refers to any amino acid. Nucleic acid sequences encoding signal sequence I and acidic sequence I are specifically contemplated, as are nucleic acid sequences complementary to such nucleic acid sequences.

25

In an embodiment of the invention there is provided the use of signal peptide II, III, IV, V or a nucleic acid encoding same, in the identification or amplification of hepcidins.

30

Signal Peptide II

MKXXXXXAXXVXXVL

Signal Peptide III

MKTFSVAV

Signal Peptide IV

MKTFSVAVTVAVVLXFICIQSSA

5 Signal Peptide V

MKTFSVAVAV (T/V) (L/V) VLA (F)_n(V/C) (C/M) (I/F) (Q/I) X (X)_m S (S/T) AV P
F XXV,

Wherein n is 0 or 1 and m is 0 or 1.

- 10 In an embodiment of the invention there is provided the use of prosequence I, Prosequence II or a nucleotide sequence encoding same or complementary to one encoding same in the identification or amplification of hepcidins.

Prosequence I

15 PEVQXLEEAXSXDNAAAEHQE

Prosequence II

PFX XVX(X)_n (L/T) EEV (E/G) (G/S) XD (T/S) PV (A/G) XHQ,

Wherein n is 0 or 1,

20

In an embodiment of the invention there is provided the use of HcPA3b3' and/or HcSal3' or a nucleotide sequence encoding same or complementary to one encoding same in the identification or amplification of hepcidins.

25

HcPa3b 3' 3'ACAACCTCGTCCTTAGG5'

HcSal 3' 3'ACGCCCCGTCCAGGAAT5'

Non-limiting Examples Of Uses

- 30 Antimicrobial peptides are useful in the treatment and/or prevention of infection in a variety of subjects, including fish, reptiles, birds, mammals, amphibians and insects.

Antimicrobial peptides are also useful for reducing bacterial growth and/or accumulation on surfaces. This is of particular benefit in the food industry where antimicrobial peptides can be used for coating surfaces used in the processing, preparation, and/or packaging of food.

5

Antimicrobial peptides disclosed herein can be administered in a variety of ways. In some instances, oral administration will be desirable. Some types of oral administration will be improved by encapsulation of the peptides so as to allow their preferential release at a particular stage in digestion. In some instances it will be desirable to include pre and/or pro sequences in the administered peptide (for example to improve stability or modulate activity). The pre and/or pro sequences can be cleaved off by endogenous proteases at the appropriate stage. Peptides may be administered by inhalation where the subject breathes air or by addition to water for gilled subjects. Administration by injection will in some cases be desirable. Peptides may be injected into any number of sites. In some cases intravenous injection will be desired. In some instances injection directly into or adjacent to the site of infection or potential infection will be desired. In some instances topical administration will be desired. Where the presence of the antimicrobial peptide is desired at a remote and specific site, or where the peptide will be desired for a prolonged period of time, gene therapy may be used to provide expression of one or more antibacterial peptides in the tissue(s) of concern.

Where the subject is a cultured or domesticated creature such as a fish, bird or non-human mammal, production of a transgenic variety which expresses one or more antibacterial peptides may be desired. Methods for producing transgenic animals are well known. (See for example Mar.Biotechnol.4: 338,2002).

A variety of antimicrobial peptides are contemplated and fall within the scope of the invention. By way of non-limiting example, peptides comprising the following amino acid sequences or a sequence at least 80% or 90% homologous thereto, and nucleic acid sequences encoding them are specifically contemplated:

- i) GW(G/K)XXFXK
- ii) GXXXXXXXXHXGXXIH
- iii) FKCKFCCGCCXXGVCGXCC

- iv) CXXCCNCC (K/H) XKGCGFCCCKF
- v) FKCKFCCGCRGXXCGLCCKF
- vi) XXXCXXCCNXXGCGXCCKX

5 Other specific, non-limiting examples of antimicrobial sequences of interest can be found in Tables 4 and 11.

Antimicrobial peptides of the invention may be modified. Such modifications may in some instances improve the peptides' stability or activity. Examples of modifications specifically contemplated include:

- 10 - conservative amino acid substitutions (acidic with acidic, basic with basic, neutral with neutral, polar with polar, hydrophobic with hydrophobic, etc.)
- addition of positively charged amino acids (lysine, arginine, histidine) at either or both ends
- replacement of amino acids with others unlikely to result in structural
- 15 changes including D-amino acids and peptidomimetics
- deletion of one or more amino acids
- modifications at C-terminal or N-terminal ends, including methyl esters and amides
- cyclised versions of the peptides (which may result in increased stability
- 20 without adversely affecting activity)

Examples – Methods

Fish Rearing

Winter flounder larvae were reared as described (Douglas, Gawlicka et al. 25 1999), the disclosure of which is incorporated herein by reference. Saint John River stock Atlantic salmon (*Salmo salar* L.) were maintained in single-pass, heated, dechlorinated fresh water at 12°C in the Dalhousie University Aquatron facility in Halifax, Nova Scotia. All fish were euthanised with an overdose of tricaine methanesulfonate (MS 222, 0.1 g L⁻¹, Argent Chemical Laboratories, Inc., Redmond, 30 WA, USA) prior to sampling. All animal procedures were approved by the Dalhousie University Committee for Laboratory Animals and the National Research Council - Halifax Local Animal Care Committee.

Bacterial Challenge

Aeromonas salmonicida subsp *salmonicida* strain A449 (Trust et al. 1983) was cultured to mid-logarithmic growth in Tryptic Soy Broth (TSB) at 17°C. The absorbance at 600nm of the bacterial suspension was determined and the bacteria were resuspended to approximately 5×10^7 cfu mL⁻¹ in sterile Hanks Balanced Salt Solution (HBSS). Three salmon (200g each) were anaesthetised with 50 mg L⁻¹ TMS, injected intraperitoneally with 2.5×10^6 cfu bacteria in 50 µL HBSS and allowed to recover in fresh water. Uninjected fish from the same cohort were maintained in separate tanks as controls. Three days post-injection, control and infected salmon were euthanised as described above and samples of tissues removed. Blood was drawn from the caudal vein into a heparinised container. To confirm that the fish were positive for *A. salmonicida*, the posterior kidney of both infected and control fish were swabbed and used to inoculate tryptic soy agar (TSA) that was incubated at room temperature overnight. Atlantic halibut tissue samples were obtained from a bacterial challenge study performed at Bedford Institute of Oceanography, Dartmouth, Nova Scotia.

Sampling

Tissues (oesophagus, stomach, pyloric caecae, liver, spleen, intestine, anterior kidney, posterior kidney, gill, skin, ovary, rectum, heart, muscle and brain) were removed into RNALater (Ambion, Austin, TX, USA) and kept at -80° C until used. Samples of winter flounder larvae at different stages and juveniles were rinsed in RNALater (Ambion, Austin, TX, USA), transferred into 1.5 ml Eppendorf tubes containing 0.5-1.25 ml RNALater, and kept at -80° C until used.

Pleurocidins

The general approach followed is shown in Figure 24

Isolation of pleurocidin cDNA

A cDNA library constructed from winter flounder skin (Gong et al 1996) was screened using degenerate oligonucleotides (PleuroA, PleuroB; Table 1). The library was plated at 80,000 phage/plate and duplicate lifts to HyBond filters were made of each of eight plates. A mixture of radioactively end-labelled PleuroA and PleuroB

probes was hybridised with the filters at 50° C using standard procedures, and the filters were washed in 1X SSC/0.1% SDS at 50° C for 45 min. Plaques that showed matching hybridization signals on both duplicate filters were picked and the library rescreened until 100% purity of the recombinant plaques was obtained. Two recombinants were completely sequenced using an ABI373 stretch automated sequencer and the AmpliTaqFS Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA). Sequence data were analyzed using Sequencher (Gene Codes, Inc., Ann Arbor, MI, USA) and DNA Strider. The amino-terminal signal sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). The Helical Wheel routine of the GCG package (http://www.gcg.com) was used to model the helical structure of the predicted antimicrobial peptide sequences.

Genomic PCR

Genomic sequences were amplified using two sets of primers specific to the winter flounder pleurocidin cDNA (PL1/PL2 and PL5'/PL3'; Table 1; Fig. 1). The amplification conditions were: 1 min at 94° C; 35 cycles of 30 s at 94° C; 30 s at 52° C, 90 s at 72° C; and 2 min at 72° C, and products were resolved on a 1% agarose gel. Bands were excised from the gel, extracted using Gene-Clean (Bio101, La Jolla, CA, USA) and cloned into the Topo TA2.1 vector (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturers. Several isolates from each transformation were sequenced and analyzed as described above. Intron positions were identified by comparison with the cDNA sequence.

Identification of additional winter flounder pleurocidin-like sequences by RT-PCR

Total RNA was isolated from winter flounder skin and intestines substantially as described in Douglas, Gawlicka *et al* (1999). Reverse transcription of 2 µg of total RNA was performed using the RETROScript kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendation. PCR was performed using PL3' and a primer corresponding to the amino terminus of the precursor polypeptide (PL5'; Table 1). The amplification conditions were: 1 min at 94° C; 32 cycles of 30 s at 94°

C, 30 s at 50° C, 90 s at 72° C; and 2 min at 72° C and products were resolved on a 2% NuSeive gel. Bands were excised, cloned and sequenced as described above.

Identification of additional pleurocidin-like sequences from different tissues

- 5 Tissue-specific expression of pleurocidin was investigated by northern analysis using polyadenylated RNA (500 ng) from adult skin, liver, ovary, muscle, spleen, pyloric caeca, stomach and intestine. The entire insert from the cDNA clone corresponding to WF2 was radioactively labelled and incubated with the blot overnight at 60° C in UltraHyb hybridisation solution (Ambion, Austin, TX, USA).
- 10 The blot was washed to a stringency of 50° C in 1X SSC/0.1% SDS for 1 h before exposure to X-ray film. RT-PCR was also employed using primers specific to WF1, WF1a, WF2, WF3, WF4, WFYT and WFX (Table 2) to assay expression of the different pleurocidin-like variants in various tissues. The conditions used were as described in the preceding paragraph except that the annealing temperature was 52 °
- 15 C.

Identification of additional pleurocidin-like sequences from different developmental stages

- 20 Two larval time series were used to assess developmental expression of pleurocidin-like genes. In the first, RNA was isolated from pooled samples of twenty whole larvae (5 and 13 dph), ten whole metamorphosing larvae (20 dph) and newly metamorphosed larvae (27 dph), gut tissue of two juveniles (41 dph), skin from the upper and lower side of adult fish and tissue from adult upper and lower intestine. RNA was isolated as described (Douglas, Gawlicka et al. 1999), the disclosure of
- 25 which is incorporated herein by reference, and the assays were performed using the primers PL5' and PL2 and conditions described above for RT-PCR. Amplification of the actin mRNA was performed as previously described (Douglas, Bullerwell et al. 1999), the disclosure of which is incorporated herein by reference, to confirm the steady level of expression of a housekeeping gene and to provide an internal control
- 30 for pleurocidin expression. In the second larval time series, RNA was isolated from pooled samples of twenty whole larvae (hatch, 5 and 9 dph), ten whole larvae (15, 20, 25, 30 and 36 dph) and gut tissue of two juveniles (41 dph). Assays were performed using primers specific to WF1, WF1a, WF2, WF3, WF4, WFYT and WFX (Table 2)

to determine expression of the different pleurocidin-like variants at different stages of development. The conditions used were as described in the preceding paragraph.

Southern analysis

5 Southern analysis of *Bam*HI- and *Sst*I-digested genomic DNA from winter flounder, three other flatfish (American plaice *Hippoglossoides platessoides* Fabricius, Atlantic halibut *Hippoglossus hippoglossus* L. and yellowtail flounder *Pleuronectes ferruginea* Storer), haddock (*Melanogrammus aeglefinus* L.), pollock (*Pollachius virens* L.) and smelt (*Osmerus mordax* Mitchill) was performed
10 sequentially using the entire inserts from genomic clones corresponding to WF1, WF2, WF3 and WF4 as probes. Hybridisations were performed overnight at 65° C as previously described (Douglas, Gallant et al. 1998), the disclosure of which is incorporated herein by reference, and the blots were washed at 65° C in 0.5X SSC/0.1% SDS for 1 h and exposed to X-ray film. Blots were stripped by incubating
15 twice in boiling 0.5% SDS and checked for residual signal by exposure to X-ray film overnight.

Identification of additional pleurocidin-like sequences from other fish species

Total RNA was isolated from skin and intestine of yellowtail flounder, witch
20 flounder and Atlantic halibut and reverse-transcribed as described above (RT-PCR analysis). Total genomic DNA was isolated from milt of yellowtail flounder, witch flounder, American plaice, Atlantic halibut and tissue samples of Petrale sole, C-O sole, English sole, Starry flounder, European plaice, Greenland halibut and Pacific halibut. Two sets of primers specific to the winter flounder pleurocidin cDNA
25 (PL1/PL2 and PL5' /PL3'; Table 1; Fig. 1) were used and the amplification conditions were: 1 min at 94° C, 32 cycles of 30 s at 94° C; 30 s at 50° C, 90 s at 72° C; and 2 min at 72° C. Products were resolved on a 2% NuSeive gel, bands excised, cloned and sequenced as described above.

30 Figure 1 is a textual and graphical depiction of WF2 pleurocidin from winter flounder A. Nucleotide sequence of cDNA for pleurocidin from winter flounder isolated from the skin library. The positions of primers used for PCR are underlined and the deduced amino acid sequence is shown in upper case letters below the

nucleotide sequence. Arrows indicate the mature 5' and 3' termini of the pleurocidin peptide and diamonds indicate the positions of introns. The single *SstI* restriction endonuclease site (GAGCTC) and the putative polyadenylation site (aataaa) are indicated in boldface. *B.* Hydrophobicity plot of predicted pleurocidin polypeptide WF2 constructed using the Kyte-Doolittle option of DNA Strider (Marck 1992). The borders of the mature pleurocidin are indicated by vertical arrows. *C.* Diagrammatic representation of helical structure of predicted pleurocidin polypeptide WF2 constructed using the Helical Wheel routine of GCG. Hydrophobic residues and glycines are boxed and polar residues are not. The first amino acid (G) of the mature polypeptide is found at the top of the wheel.

Identification of pleurocidin-like sequences in the winter flounder genome

A winter flounder genomic λ -GEM library was screened using a radioactively labeled probe for pleurocidin (WF2; Douglas et al., 2001). Four clones were picked and replated until 100% purity was achieved. The clones were mapped using *BamHI*, *SstI*, *XhoI* and *Eco RI* and two clones (λ 1.1 and λ 5.1) that differed in restriction pattern were selected for sequencing. Both clones were completely sequenced using an ABI373 stretch automated sequencer and the AmpliTaqFS Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, CA, USA. Transcription factor binding sites were identified using WWW Signal Scan (<http://bimas.dcrtnih.gov/molbio/signal/>) with the TransFac and TFD databases and promoters were detected using the eukaryotic promoter prediction by neural network software available at the Baylor College of Medicine (<http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html>).

25 Hepcidins

The general approach followed is depicted in Figure 24

Molecular Characterisation of Hepcidin cDNAs

Eight ESTs showing high similarity to human hepcidin were identified from the winter flounder EST database (Douglas, Gallant et al. 1999) and four from the Atlantic salmon database (Douglas, Tsoi et al. 2002). Using these sequences to screen dbEST, BLASTX analysis revealed two related sequences from Japanese flounder (C23298.1 and C23432.1), one sequence from rainbow trout (AF281354_1) and five

identical sequences from medaka (AU178966, AU179222, AU179314, AU179768 and AU180044). Sequence data were analyzed using Sequencher (Gene Codes, Inc., Ann Arbor, MI, USA) and DNA Strider (Marck 1992). Alignments and similarity matrices were calculated using ClustalW (Thompson, Higgins et al. 1994) and graphically visualised using SeqVu (Garvan 1996). The on-line servers PSORT (<http://PSORT.nibb.ac.jp>), Compute pI (<http://expasy.hcuge.ch/cgi-bin/pi> tool), and Network Protein Sequence @analysis (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl) were used to predict N-terminal signal sequences, pI and secondary structure, respectively. The secondary structure prediction program utilized seven different algorithms (for details, see web site) and provided a consensus prediction based on these results.

Southern Hybridisation

Total genomic DNA was prepared from winter flounder (*Pleuronectes americanus*), yellowtail flounder (*Pleuronectes ferruginea*), witch flounder (*Glyptocephalus cynoglossus*), Japanese flounder (*Paralichthys olivaceus*), American plaice (*Hippoglossoides platessoides*), Atlantic salmon (*Salmo salar*), haddock (*Melanogrammus aeglefinus*), smelt (*Osmerus mordax*), hagfish (*Eptatretus burgeri*), tiger shark (*Scyliorhinus torazame*) and white sturgeon (*Acipenser transmontanus*) as previously described (Douglas, Bullerwell et al. 1999), the disclosure of which is incorporated herein by reference. DNA (7.5 μ g) was digested with *Sst*I according to the manufacturer's recommendations and the fragments resolved on a 1% agarose gel. A 104 bp probe corresponding to amino acid residues WMENPT...GCGFCC of Type I winter flounder hepcidin was labeled using the DIG Labelling Kit (Roche Applied Science, Laval, PQ, Canada) and hybridized to the membrane for 2h at 42 °C using the Easy Hyb kit (Roche Applied Science, Laval, PQ, Canada). The membrane was washed in 0.2X SSC at 65 °C and signal detected using the DIG Luminescent Detection Kit (Roche Applied Science, Laval, PQ, Canada).

Identification of additional hepcidin-like sequences by RT-PCR

Primers were designed based on the cDNA sequences determined in this study (Table 3). Amplification of actin mRNA was performed to confirm the steady-state level of expression of a housekeeping gene and provide an internal control for the hepcidin gene expression analyses. Controls were performed using single primers to

eliminate single primer artifacts and without reverse transcription to eliminate amplification products arising from contaminating genomic DNA.

5 Total RNA was isolated from tissues of uninfected adult winter flounder and uninfected and infected adult salmon and halibut using the RNAWiz Kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. Tissues were homogenized using a 7mm generator on a Polytron standard rotor stator homogenizer (Kinematica). In addition, RNA was isolated from pooled samples of twenty whole larvae (hatch, 5 and 9 dph), ten whole larvae (15, 20, 25, 30 and 36 dph), gut tissue of two juveniles (41 dph) and adult winter flounder liver. To eliminate contaminating
10 DNA, the Ambion DNA-free™ protocol was used as directed. Briefly, 4 units of DNase 1 was added to the resuspended RNA and incubated for 1 hour at 37°C. After incubation, DNase Inactivation Reagent was added to remove the enzyme and RNA concentrations were determined using a Beckman DU-64 Spectrophotometer.

15 First strand cDNA was synthesized from 1 µg of total RNA using the RetroScript kit (Ambion, Austin, TX, USA) and aliquots of the reaction products were subjected to PCR using rTaq polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or the Advantage2 PCR kit (Clontech, Palo Alto, CA, USA). The primers and annealing temperatures are listed in Table 3. The amplification conditions
20 were: 1 min at 95° C; 32 cycles of 15 s at 95° C; 30 s at the annealing temperature, 30 s at 68° C; hold at 4° C. Amplification products were resolved on a 2% NuSieve agarose gel with a 100 bp ladder as a marker (Gibco BRL, Gaithersburg, MD, USA) and the amount of each product was quantified using a GelDoc 1000 video gel documentation system (BioRad, Mississauga, Ont., Canada) with the Multianalyst
25 software.

Identification of additional hepcidin-like sequences from other fish species

Total RNA was isolated from liver and spleen of bacterially challenged Atlantic halibut and Atlantic salmon and reverse-transcribed as described above (RT-
30 PCR analysis). Two sets of primers were used (see legend, Fig. 2) and the amplification conditions were: 2 min at 94° C; 32 cycles of 30 s at 94° C; 30 s at 52° C, 30 s at 72° C; and 2 min at 72° C. Products were resolved on a 2% NuSeive gel, bands excised, cloned and sequenced as described above.

Prediction of active cationic peptide sequences

The mature peptide sequences from Figure 3 (pleurocidin-like peptide sequences deduced from nucleotide sequences of genes and PCR products amplified from fish tissues) constituted the basis of sequence selection. Generally, upon extensive sequence analysis, those peptides that possessed a net positive charge and had their hydrophilic and hydrophobic residues well-separated in models were produced. Also, generally those peptide genes that were likely to be expressed (possessed promoters) were used, although pseudogenes were also included in the panel. The exact start/end residues were decided upon based on several factors listed below. In most cases the N-terminus of the mature peptide was well defined, since it followed directly the conserved signal peptide region, and aligned well with other mature peptides. Wherever a straightforward determination on the N-terminal amino acid was not possible, an attempt was made to preserve GW or GF at the N-terminus, as this is frequently encountered among cationic peptides. In addition, two versions of WF1a (NRC-2 and NRC-3) were produced: one contained N-terminal GRRKRK, and the other did not. In some cases the C-terminus of the mature peptide was also well defined, since it was followed directly by a conserved acidic propiece. However significant ambiguity as to the C-terminal amino acid existed among many peptides. Generally, two rules were followed in deciding upon C-terminal amino acids: (1) wherever glycine appeared at or near the C-terminus, it was considered to be a precursor for carboxy-terminus amidation; (2) large numbers of negatively charged amino acids near the C-terminus were generally considered to be a part of the propiece and not the mature active peptide, and were not included in the sequence.

25

All antimicrobial peptides used in this study were synthesized by N-(9-fluorenyl) methoxy carbonyl (Fmoc) chemistry at the Nucleic Acid Protein Service (NAPS) unit at the University of British Columbia. Peptide sequences are shown in Table 4. Peptide purity was confirmed by HPLC and mass spectrometry analysis in each case. In the case of NRC-7 further purification by RP-HPLC was performed until homogeneity of the sample was obtained.

30

Bacterial Strains and *Candida albicans*

All strains used in this study are listed in Table 5. Most non-fish bacterial strains as well as *Candida albicans* were grown at 37°C in Mueller-Hinton Broth (MHB; Difco Laboratories, Detroit), while the fish bacteria were maintained at 16°C in Tryptic Soy Broth (TSB; Difco, 5g/l NaCl). All strains were stored at -70°C until they were thawed for use and sub-cultured daily. The following strains, *Pseudomonas aeruginosa* K799 (parent of Z61), *Pseudomonas aeruginosa* Z61 (antibiotic supersusceptible), *Salmonella typhimurium* 14028s (parent of MS7953s), *Salmonella typhimurium* MS7953s (defensin supersusceptible), as well as *Staphylococcus epidermidis* (human clinical isolates) and methicillin-resistant *Staphylococcus aureus* (MRSA; isolated by Dr. A. Chow, University of British Columbia) have been kindly donated by Prof R.E.W. Hancock, University of British Columbia.

Escherichia coli strain CGSC 4908 (*his-67*, *thyA43*, *pyr-37*), auxotrophic for thymidine, uridine, and L-histidine (Cohen *et al.*, 1963) was kindly supplied, free of charge, by the *E.coli* Genetic Stock Centre (Yale University, New Haven, CT). MHB supplemented with 5 mg/L thymidine, 10 mg/L uridine and 20 mg/L L-histidine (Sigma Chemical Co., St. Louis, MO), was used to grow *E.coli* CGSC 4908 unless otherwise specified.

Two field isolates of the salmonid pathogen *Aeromonas salmonicida* are from the IMB strain collection.

Minimum Inhibitory Concentrations

The activities of the antimicrobial peptides were determined as minimal inhibitory concentrations (MICs) using the microtitre broth dilution method of Amsterdam (Amsterdam, 1996), as modified by Wu and Hancock (1999). Serial dilutions of the peptide were made in water in 96-well polypropylene (Costar, Corning Incorporated, Corning, New York) microtiter plates. Bacteria or *C. albicans* were grown overnight to mid-logarithmic phase as described above, and diluted to give a final inoculum size of 10⁶ cfu/ml. A suspension of bacteria or yeast was added to each well of a 96 well plate and incubated overnight at the appropriate temperature. In the case of *E. coli* CGSC 4908, supplemented MHB was used. Inhibition was defined as growth lesser or equal to one-half of the growth observed in control wells,

where no peptide was added. Three repeats of each MIC determination were performed.

Killing assays

5 Survival of bacteria and *C. albicans* upon exposure to selected peptides applied at their minimal inhibitory concentrations (MICs) and ten times their MICs was measured using standard methodology. The test organisms were grown in MHB and exposed to the peptides. At the specified time intervals equal aliquots were removed from the cultures, plated on MHB plates, and the resulting colonies were
10 counted. Percentage survival was plotted against time on a logarithmic scale. Two repeats of each experiment were performed.

Preparation of a Synthetic Antimicrobial Peptide

Prediction of active cationic peptide sequences.

15

The mature peptide sequences from Figure 3 (pleurocidin-like peptide sequences deduced from nucleotide sequences of genes and PCR products amplified from fish tissues) constituted the basis of sequence selection.

Sequences were selected for peptides that possessed a net positive charge and
20 had their hydrophilic and hydrophobic residues well separated spatially in models that were produced specifically:

- a) In order to estimate the net charge K and R were assumed to have the value of +1, H of +1/2, D and E of -1, and C-terminal amidation was
25 counted as an additional +1.
- b) The EMBOSS Pepwheel and Pepnet internet tools available through an NRC mirror site (<http://bioinfo.pbi.nrc.ca:8090/EMBOSS/index.html>) were used to analyse the separation of hydrophilic and hydrophobic residues in helical wheel and helical net models.

30

Also, generally those peptide genes that were likely to be expressed (possessed promoters, were transcribed, etc.) were produced, although pseudogenes were also included in the panel.

The exact start/end residues were decided upon based on several factors:

- a) In most cases the N-terminus of the mature peptide was well-defined, since it followed directly the conserved signal peptide region, and aligned well with other mature peptides.
- 5 b) Wherever a straightforward determination on the N-terminal amino acid was not possible, an attempt was made to preserve GW or GF at the N-terminus, as this is frequently encountered among cationic peptides.
- c) In addition, two versions of WF1a (NRC-2 and NRC-3) were
10 produced: one contained N-terminal GRRKRK, and the other did not; this was done because it was hypothesized that the presence of the highly positively charged GRRKRK would improve activity.
- d) Although in some cases the C-terminus of the mature peptide was also well defined, since it was followed directly by a conserved acidic
15 propiece, significant ambiguity as to the C-terminal amino acid existed among many peptides. Generally, two rules were followed in deciding upon C-terminal amino acids:
1. wherever glycine appeared at or near the C-terminus, it was considered to be a precursor for
20 carboxy – terminus amidation;
 2. large numbers of negatively charged amino acids near the C-terminus were generally considered to be a part of the propiece and not mature active peptide and were not included in the sequence.
- 25 Peptides produced according to the above steps are screened for antimicrobial activity *in vitro* by standard means. Those peptides showing *in vitro* antimicrobial activity are useful as antimicrobial peptides for use *in vivo* and for the treatment of surface, etc.

Examples - Results

Pleurocidins

30 **cDNA sequence**

The two clones isolated from the skin cDNA library were identical in sequence to each other and to the genomic PCR product WF2 after introns were removed (see

below). They contain 356 bp and encode an open reading frame of 68 amino acids (Fig. 1A). There is a 5'-untranslated region of 26 bp and a 3'-untranslated region of 84 bp, excluding the polyA tail. A canonical polyadenylation signal AATAAA is found 22 bp upstream of the polyA tail. The first 22 amino acids of the open reading frame form a highly hydrophobic domain (Fig. 1B) predicted to be a signal peptide with a cleavage site that precisely matches the amino terminus of the mature pleurocidin. The predicted amino acid sequence of residues 23-47 exactly matches the published amino acid sequence of mature pleurocidin (arrows, Fig. 1A). The mature peptide can assume an amphipathic helix that contains a predominance of positively charged amino acids on one face and hydrophobic amino acids on the other (Fig. 1C). The carboxy-terminal 21 amino acids form a negatively charged domain that is not present in the mature pleurocidin, confirming the recent report of Cole et al. (2000).

Genomic PCR

Four distinct bands (WF1-4) were amplified using primers PL5' and PL3' (Fig. 4). Sequence analysis of each product was consistent with the sizes of the bands and verified that each amplification product was different (Table 6). Two distinct bands were amplified using primers PL1 and PL2 that corresponded to WF2 and WF4 containing additional upstream and downstream sequence (data not shown). When the intron sequences were removed, the sequence of WF2 exactly matched that of the pleurocidin cDNA clone isolated from the skin library (Fig. 1A).

Figure 4 is a depiction of the results of PCR amplification of pleurocidin-like sequences from winter flounder genomic DNA. Amplification products (P) were resolved on a 1 % agarose gel using the 100 bp ladder as molecular weight markers (M). Products visible as distinct bands are labeled WF1 (900 bp), WF2 (810 bp), WF3 (650 bp) and WF4 (510 bp).

All four of the pleurocidin-like genes contained two introns within the coding sequence and three of the genes showed identical intron locations (WF1, WF2 and WF4). However, the position of the second intron in WF3 occurred upstream of those of the other genes, resulting in a shorter second exon and longer third exon. The sizes and sequences of the introns varied among the four pleurocidin genes (Table 6). Evidence from the two more extensive genomic sequences of WF2 and WF4 obtained

using primers PL1 and PL2 indicates that a third intron immediately upstream of the initiation codon is also a feature of this gene family (Fig. 5). This was also noted for the genomic sequence reported by Cole et al (Cole, Darouiche et al. 2000).

5 An alignment of the predicted amino acid sequences is shown in Fig. 6. The positions of the introns (indicated by vertical arrows) were determined by comparison with the corresponding RT-PCR and cDNA-derived sequences. The positions of the mature peptide were determined by comparison with the published amino acid sequence of pleurocidin (Cole, Weis et al. 1997). All of the predicted mature
10 polypeptides could assume amphipathic α -helical structures similar to that shown in Fig. 1C, although the positively charged portions were not as striking in WF1 and WF3 as in WF2 and WF4 (data not shown).

Figure 5 describes extended genomic sequence of WF4 obtained by PCR using
15 primers PL1/PL2. Introns are indicated in lower case and coding sequence in upper case. The positions of the primers PL1 and PL2 used for PCR are underlined.

Figure 6 describes Alignment of predicted polypeptide sequences of five winter flounder pleurocidin family members. Large vertical arrows indicate the positions
20 where introns were found in the genomic sequences. The second intron of WF3, indicated by a small vertical arrow, is found more upstream than those of the other genes. The predicted polypeptide sequences of dermaseptin B1 (Amiche et al. 1994) and ceratotoxin B (Marchini et al. 1995) are shown below the pleurocidin family members. Boxed amino acids are shared by half of the sequences.

25

Identification of additional pleurocidin-like sequences from different tissues

Northern analysis was only able to detect pleurocidin transcripts in skin (data not shown). However, the more sensitive RT-PCR assay indicated that pleurocidin was also expressed in other tissues, particularly gill and gut. Using primers PL5' and
30 PL3', two bands were obtained from winter flounder skin (265 and 175 bp) and two from intestine (215 and 175 bp). Sequence analysis of several clones of each size showed that the 265 bp winter flounder skin clones corresponded to the genomic sequence of WF1 when intron sequences were removed (Table 7). Five of the 175 bp clones from skin and two of the 175 bp clones from intestine corresponded to the

genomic sequence of WF2. This is consistent with results of northern analysis using the cDNA clone corresponding to the WF2 probe that showed hybridisation only to 200-nucleotide mRNA from the skin (data not shown). On the other hand, nine of the 175 bp clones from intestine and four of the 175 bp clones from skin corresponded to the genomic sequence of WF3. No RT-PCR products were obtained that corresponded to WF4. All seven of the 215 bp intestine clones corresponded to a novel family member (WF1a) not represented by any of the winter flounder genomic sequences determined in this study.

Using primers specific to each of the pleurocidin-like variants reported above, as well as to additional pleurocidin-like variants identified on Lambda clones, we were able to demonstrate that different variants were expressed in different tissues (Fig. 7). WF2, WF3 and WFYT showed the expression in the widest distribution of tissues, whereas WF1 and WF4 were expressed in mainly in the gill and skin, and WFX was only expressed in the skin. Transcripts of WF1a could not be detected in any tissue.

Figure 7 describes the expression of specific pleurocidin-like genes in different tissues of winter flounder. Tissues were esophagus (E), pyloric stomach (PS), cardiac stomach (CS), pyloric caeca (PC), liver (L), spleen (SP), intestine (I), rectum (R), gill (G), brain (B) and skin (SK). Markers (M) were the 100 bp ladder. Primers were specific to each pleurocidin variant (Table 2)

Identification of additional pleurocidin-like sequences from different developmental stages

Using primers PL5' and PL2 (Table 1) from highly conserved regions of the pleurocidin-like peptides, low levels of transcripts were evident at 5 dph and increased during development (Fig. 8). Strong signals were obtained from adult skin and weak signals from intestinal tissue. Expression of the housekeeping gene, actin, was relatively constant throughout development.

Using primers specific to each of the pleurocidin-like variants reported above, as well as to additional pleurocidin-like variants identified on Lambda clones, it was demonstrated that different variants were expressed at different times during development (Fig. 9). WFX transcripts were only detectable at 20 dph, and WF2,

WF3 and WFYT were detectable in premetamorphic larvae and metamorphic juveniles. No expression of WF1 and WF4 was detectable at any stage of development.

5 Figure 8 describes Reverse transcription-polymerase chain reaction assay of pleurocidin expression. Samples are from larvae (5 and 13 dph), metamorphosing larvae (20 dph), newly metamorphosed larvae (27 dph), juveniles (41 dph), skin from the lower (LS) and upper side (US) of the fish and tissue from the lower (LI) and upper (UI) intestine. Primers specific for pleurocidin (panel A) and actin (panel B)
10 were used.

 Figure 9 describes Expression of specific pleurocidin-like genes during winter flounder larval development. Samples are from larvae (5, 9 and 15 dph), metamorphosing larvae (20 dph), newly metamorphosed larvae (25, 30 and 36 dph)
15 and juveniles (41 dph). Controls using the 5' or 3' primers alone and with no template (NT) are also shown. Primers were specific to each pleurocidin variant (Table 2).

Southern analysis

 Positive signals were specific to flatfish DNA using the WF1, WF2, WF3 and
20 WF4 genomic probes (Fig. 10). No signals were detected with haddock, pollock or smelt DNA (data not shown). All four probes showed hybridisation to common *SstI* and *BamHI* bands from the DNAs of all four flatfish, indicating that the genes are clustered on these genomes. The sizes of the hybridising fragments from the winter flounder digest are given in Table 8.

25

 Figure 10 describes Southern analysis of pleurocidin genes of winter flounder (WF), yellowtail flounder (YF), American plaice (AP) and Atlantic halibut (AH). Total genomic DNA (7.5 µg) was digested with *BamHI* (B) or *SstI* (S) and the fragments resolved on a 1.0% agarose gel. The blot was hybridized successively with
30 probes corresponding to WF1, WF2, WF3, and WF4. Markers (M) are lambda DNA digested with *StyI* (24.0, 7.7, 6.2, 3.4, 2.7, 1.9, 1.4, 0.9 Kb).

Identification of additional pleurocidin-like sequences from other fish species

An alignment of the deduced amino acid sequences of pleurocidin-like peptides from American plaice, yellowtail flounder, witch flounder and Atlantic halibut is shown in Fig. 3. Sequences were obtained from genomic DNA of Petrale sole, C-O sole, English sole, starry flounder, European plaice, Greenland halibut and Pacific halibut. High conservation is present in the signal peptide and acidic propiece regions, whereas the portion corresponding to the mature peptide shows much more variability.

Figure 3 describes Alignment of pleurocidin-like peptide sequences deduced from nucleotide sequences of genes and PCR products amplified from skin and/or intestine of the following species: winter flounder (WF), yellowtail flounder (YF), witch flounder (GC), American plaice (AP) and Atlantic halibut (AH). Specific non-limiting examples of pleurocidin-like sequences identified are shown in Table 4. Non-limiting examples of cDNA and/or genomic sequences are provided in Appendix L

Identification of pleurocidin-like sequences in the winter flounder genome

Two clones containing fragments of 12.5 and 15.6 kb, respectively, were isolated from a genomic library from winter flounder. The 12.5 kb fragment encoded the gene corresponding to WF2 and two pseudogenes. The 15.6 kb fragment encoded the gene corresponding to WF1, one pseudogene and two previously undescribed pleurocidin-like sequences referred to as WFX and WFYT. A schematic of the gene arrangement is shown in Fig. 11. Scanning of the sequences upstream of the coding sequence revealed a canonical eukaryotic promoter, TATA and CAAT boxes as well as highly conserved sites for several transcriptions factors including NF-IL6, AP1 and α -interferon (Fig. 12). No promoter sequences were identified upstream of pseudogenes.

Figure 12 describes Locations of transcription factor binding sites upstream of pleurocidin genes and pseudogenes. Promoters are indicated by hatched boxes, introns by solid boxes and genes and exons by stippled boxes.

Prediction and assessment of antimicrobially active peptide sequences

The minimal inhibitory concentrations of the chemically produced peptides against a wide range of bacterial pathogens and *C. albicans* were determined and are shown in Table 9. Generally speaking many peptides showed the ability to inhibit the growth of a broad spectrum of bacterial pathogens and *C. albicans*. Particularly good examples of peptides with a broad spectrum of antimicrobial activity are the three peptides derived from American plaice (NRC-11, NRC-12, and NRC-13) and three peptides derived from witch flounder (NRC-15, NRC-16, and NRC-17). Of those, NRC-15, NRC-13, and NRC-12 showed ability to kill methicillin-resistant *S. aureus* (Fig. 13), *P. aeruginosa* (Fig. 14) and *C. albicans* (Fig. 15), respectively.

Figure 13 describes Survival of a Gram-positive bacterium (methicillin-resistant *Staphylococcus aureus* - MRSA) upon exposure to NRC-15 at its minimal inhibitory concentration (MIC) and ten times its MIC. *S. aureus* was grown in Mueller-Hinton broth and exposed to NRC-15 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

Figure 14 describes Survival of a Gram-negative bacterium (*Pseudomonas aeruginosa*) upon exposure to NRC-13 at its minimal inhibitory concentration (MIC) and ten times its MIC. *P. aeruginosa* was grown in Mueller-Hinton broth and exposed to NRC-13 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

Figure 15 describes Survival of a yeast (*Candida albicans*) upon exposure to NRC-12 at its minimal inhibitory concentration (MIC) and ten times its MIC. *C. albicans* was grown in Mueller-Hinton broth and exposed to NRC-12 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

In addition to demonstrating that pleurocidin-like peptides are active against a wide range of bacteria as well as *C. albicans*, the results indicate which factors should

preferably be considered in selecting antimicrobially active peptides from genomic sequences.

5 Firstly, a notable group of peptides with poor or no observed activities were peptides derived from pseudogenes (NRC-8, NRC-9, NRC-10). These results indicate that peptides capable of being expressed in the host organism may be better candidates for antimicrobials.

10 Secondly, the previously described N-terminal GRRKRK in WF1a (Fig. 2) proved to be a determinant of antimicrobial activity in NRC-3 as shown by the fact NRC-2 (identical to NRC-3 but missing the aforementioned fragment) was only marginally active (Table 9). This result stresses the importance of carefully selecting the start/end residues in the mature peptide, wherever these are not apparent in the original pre-pro-sequence.

15

Thus in an embodiment of the invention there is provided a group of pleurocidin-related antimicrobial peptides having the amino acid sequence GRRKRK. It will be appreciated that pleurocidin-like antimicrobial peptides lacking this sequence also exist and are specifically contemplated herein.

20

The previously described principles of: selecting positively charged peptides with good separation of hydrophilic and hydrophobic residues in helical wheel models, preserving GW or GF at the N-terminus, amidating the C-terminus where glycine was present, and cropping off clusters of acidic C-terminal amino acids were
25 successful in selecting antimicrobially active peptides.

Peptides of the invention can be used at a range of pH's, salt concentrations, and temperatures. These peptides are useful against pathogens grown in biofilms or under any other conditions for pathogen growth or culture. See for example Figure 25 in
30 which the ability of NRC-13 to kill *P. aeruginosa* K799 in 50 mM NaCl is shown. NRC-13 was added to a culture of *P. aeruginosa* supplemented with 150 mM NaCl to a final concentration of 4 µg/ml (□) or 40 µg/ml (Δ), representing the MIC and 10X MIC, respectively. A control with no peptide added is also shown (♦).

Peptides may be used alone or in combination with one or both of their pre-and pro- sequences.

Peptides of the invention have many uses, including as antibacterial, antifungal, antiviral, anti-cancer, and antiparasitic agents, including in combination with other antibiotics, anti-infectives, and chemotherapeutants as well as with each other.

Peptides can be used as immunomodulatory agents such as for wound healing, tissue regeneration, anti-sepsis, immune promoters, etc. including in combination with other agents.

The peptides can be delivered topically (including e.g., aerosols-especially for respiratory tract infections in CF patients, ointments, lotions, rinses, eyewashes, etc.), systemically (including e.g. IV, IP, IM, subcutaneously, intracavity or transdermally) and, orally (e.g. pills, liquid medication, capsules, etc.).

Delivery via encapsulation, including in liposomes, proteinoids is contemplated, as is delivery in transgenic systems involving agricultural animals and/or plants.

Peptides can be used as protective coatings on medical devices (including catheters, etc, food preparation machinery and packaging.

Examples of antibiotics which can be used together with peptides disclosed herein in aquaculture operations include: Terramycin Aqua (oxytetracycline), Romet (sulfadimethoxine and ormetoprim), and Tribissen (trimethoprim and sulfadiazine. In the hatchery, dipping in formaldehyde can be used together with peptides disclosed herein. Peptides can be used in combination with each other and/or in combination with conventional antibiotics for any of the uses described herein.

Hepcidins

Specific non-limiting examples of hepcidin sequences identified are shown in Table 11. Examples of cDNA or genomic sequences are shown in Appendix II.

Bacterial Challenge

Three days post-injection, the infected Atlantic salmon were lethargic and anorexic. On sampling, the posterior kidneys of the injected fish were positive for *A. salmonicida* whereas those of the control fish were not.

5

Molecular Characterisation of Hecpidin cDNAs

Although the winter flounder EST database contains sequences from liver, ovary, stomach, intestine, spleen and pyloric caecae cDNA libraries and the Atlantic salmon EST database contains sequences from liver, head kidney and spleen, hepcidin-like sequences were only detected in spleen and liver cDNA libraries of both fish. Four of 135 ESTs (3.0%) in the winter flounder liver library and two of 281 ESTs (0.7%) in the winter flounder spleen library encoded hepcidins. Three of 982 (0.3%) ESTs in the Atlantic salmon liver library encoded hepcidins. Five hepcidin sequences were also found in subtracted spleen (1.8%) and three in subtracted liver (0.6%) Atlantic salmon cDNA libraries that were enriched in transcripts up-regulated during infection with *Aeromonas salmonicida*. Unfortunately, since these are subtracted libraries, the inserts are only portions of the complete transcripts.

Analysis of the nucleotide sequences of Atlantic salmon hepcidin cDNAs revealed that one salmon EST (SL1-0412) was approximately 300 nucleotides longer than the other two. Furthermore, the hepcidin coding sequence was incomplete. Complete sequencing of this clone revealed the presence of two introns with standard GT/AG splice junctions (Fig. 16A). When removed, an open reading frame encoding a complete hepcidin-like peptide was obtained. Similarly, an incompletely spliced halibut transcript was amplified that still retained the second intron (Fig. 16B). Compared to mammals, the introns of salmon and probably halibut are in similar locations but of shorter length (Fig. 16C). In addition to these incompletely spliced cDNAs, we identified a winter flounder EST (WF4) that contains a large deletion relative to the other sequences that corresponded closely to the second exon of salmon and human hepcidin. Assuming the intron positions are conserved among vertebrates, this deletion could correspond to the removal of exon 2, and resulted in a peptide that differed from WF3a and WF3b in only five amino acid positions of the remaining peptide.

Figure 16 describes a Nucleotide sequence of unspliced liver cDNA encoding Type I salmonid hepcidin. Exon sequences are indicated in upper case letters and the deduced amino acid sequence is shown below the nucleotide sequence. The gt/ag intron/exon boundaries are highlighted in boldface and the polyadenylation signal (aataaa) is underlined. B. Nucleotide sequence of partially spliced cDNA from halibut spleen encoding Type I salmonid hepcidin. C. Comparison of intron/exon structure in human, mouse and salmon. Exons are represented by hatched boxes and introns by a single line (sizes in bp shown beneath).

10 The deduced amino acid sequences of five different winter flounder hepcidin cDNAs and two different Atlantic salmon hepcidins were aligned for comparison purposes with those extracted from dbEST corresponding to Japanese flounder (two), medaka (one) and rainbow trout (one), as well as the recently reported hepcidin from hybrid striped bass (Shike et al. 2002) and two from Atlantic halibut (Hb 17 and Hb 15 357). The sequences obtained from spleen and liver of Atlantic salmon (Sal2.1 and Sal8.6) and Atlantic halibut (Hb1.1, Hb5.3 and Hb7.5) by PCR are also included (Fig. 17). Human hepcidin was included as a representative of the mammals. The position of cleavage by signal peptidase was predicted by PSORT and the RX(K/R) motif typical of propeptide convertases (Nakayama 1997) was identified (vertical arrows; 20 Fig. 17). The signal peptide sequence is 22-24 amino acids and is highly conserved among all of the fish sequences. The anionic propiece is 38-40 amino acids, depending on the particular hepcidin variant. The processed hepcidins contain 19-27 amino acids and all are positively charged at neutral pH except WF2 (Table 10). Types I and III hepcidin from flatfish as well as salmon type hepcidin contain eight 25 cysteine residues in the mature peptide, which have been proposed to form four disulphide bonds. Type II winter flounder hepcidin is missing two cysteine residues, indicating that a maximum of three disulphide bonds could form. Hb357 contains only five cysteine residues and is quite different from the remaining hepcidin-like sequences. Results of secondary structure prediction methods indicated that the 30 consensus structure of fish hepcidins was mostly random coil, although short stretches of extended strand were predicted by some methods.

Figure 17 describes Alignment of winter flounder (WF1, WF2, WF3a, WF3b, WF4), Atlantic halibut (Hb1.1, Hb5.3, Hb7.5, Hb17, Hb357) and Atlantic salmon

(Sal1, Sal2, Sal2.1, Sal8.6) hepcidins with those of Japanese flounder (JFL4, JFL6), medaka, hybrid striped bass and human. A partial sequence from rainbow trout (GenBank accession AF281354_1) is also shown. The predicted positions of signal peptidase and pre-protein cleavages are indicated by arrows.

5

From Figure 17, it is apparent that all of the flatfish-type hepcidins have very similar signal peptides, which differ somewhat from the salmonid type and human hepcidin. Other novel features identified included different groups of hepcidins based on (1) number of cysteines, (2) unique insertion FKC in flatfish Type III, (3) two other locations that may contain unique insertions (4) a truncated version (Flatfish Type IV), (5) longer versions at the amino terminus.

10

Based on the alignment, it is apparent that there are at least three different groups of flatfish hepcidins distinguishable by shared insertions and deletions. WF2 and JFL6 (Flatfish Type II) share a deletion of seven amino acids near the KR cleavage site resulting in a processed peptide of 19 amino acids, whereas WF3a, WF3b, WF4, Hb1.1, Hb17, Hb5.3 and Sal8.6 (Flatfish Type III) exhibit a deletion of only four amino acids (excluding the portion corresponding to the missing exon of WF4) resulting in processed peptides of 22 amino acids. WF1 and JFL4 (Flatfish Type I) do not contain this deletion but do contain an insertion relative to all other reported hepcidins at a position adjacent to the signal peptidase cleavage site. In addition, WF1, bass and medaka share an insertion of one amino acid within the mature peptide relative to all other reported hepcidins, giving a peptide of 26-27 amino acids. WF3a and WF3b differ from each other by only one amino acid although they contain several silent substitutions and differences in the 5' and 3' untranslated regions. Hb357 represents a possible fourth class of flatfish hepcidins. The 3' untranslated regions of WF2 and WF1 are very different from those of the other hepcidin transcripts, WF2 containing a long additional portion relative to the others and WF1 being shorter and less highly conserved (Fig. 18A).

25
30

The salmonid hepcidin-like peptides fall into one group; the four reported sequences all share two deletions and differ from each other by four amino acids in the mature peptide and four amino acids in the upstream pre-protein portion. The 3'

untranslated regions of the salmon hepcidins are only moderately conserved (Fig. 18B).

Figure 18 describes Alignment of 3' untranslated regions of (A) winter flounder (WF1, WF2, WF3a, WF3b, WF4) and (B) Atlantic salmon (Sal1, Sal2) hepcidin cDNAs. Conserved nucleotides are boxed. The positions of the primers used to amplify hepcidin homologs from halibut and salmon are indicated by arrows.

Genomic Organisation of Winter Flounder Hepcidin Genes

Southern hybridization analysis of genomic DNA from a wide variety of fish with a probe corresponding to Type I hepcidin identified bands in all flatfish tested but none of the other fish species (Fig. 19). In winter flounder, two fragments of 4.3 and 4.5 kb hybridized with the probe. Two fragments of yellowtail flounder of identical size hybridized (4.3 kb) and two fragments of witch flounder genomic DNA also hybridized (4.3 and 20 kb), whereas only one fragment (4.3 kb) of the American plaice and one fragment (5.5kb) of the Japanese flounder genomic DNA hybridized.

Figure 19 describes Southern hybridization analysis of hepcidin in different fish species. *Sst*I digests of genomic DNA (7.5 µg) from hagfish (Hg), shark (Sh), white sturgeon (St), winter flounder (WF), yellowtail flounder (YF), American plaice (AP), witch flounder (Wi), Japanese flounder (JF), Atlantic salmon (AS), smelt (Sm) and haddock (Hd) were hybridized with Type I hepcidin from winter flounder. Size markers (M) are Lambda DNA digested with *Sty*I.

Identification of Hepcidin-like sequences by RT-PCR

Figure 2 describes amplification of hepcidin cDNAs from halibut and salmon liver and spleen. RNA was prepared from tissues of fish infected with a bacterial pathogen to induce expression of antimicrobial peptide genes, reverse-transcribed and subjected to PCR using the primers listed below. Actin was run as a control to show expression of a house-keeping gene. The labelling on the figure is as follows: HL - halibut liver; SL - salmon liver; HS - halibut spleen; SS - salmon spleen; M - markers. For the primers 5'U is the Universal 5' primer used in all reactions, Sal is Hc Sal (below) and WF is HcPA3b (below).

HepUniversal 5': AAGATGAAGACATTCAGTGTTGCA

HcPA3 3'B2: GTTGTTGGAGCAGGAATCC

Hc Sal: TGCTGGCAGGTCCTCAGAATTTGC

5 The results of RT-PCR assays of tissue-specific expression of the three winter flounder hepcidins are shown in Fig. 20. Type I hepcidin was abundantly expressed in the liver and, to a lesser extent, in the cardiac stomach. Type II hepcidin could not be detected in any tissues, whereas Type III hepcidin was moderately expressed in the esophagus, cardiac stomach, and liver.

10

In uninfected Atlantic salmon, Type I hepcidin was expressed at quite high levels in the liver, blood and muscle, at low levels in gill and skin, and at barely detectable levels in anterior and posterior kidney (Fig. 21A, Table 10). Type II hepcidin was expressed at barely detectable levels in the gill and skin only (Fig. 21B). However, 15 fish infected with *Aeromonas salmonicida* showed expression of both types of hepcidin in most tissues tested (see below).

RT-PCR analysis of hepcidin gene expression in winter flounder larvae of different ages is shown in Fig. 22. Transcripts of Type II hepcidins could not be detected at any stage of development, whereas Type I and Type III hepcidins were detectable in pre- 20 metamorphic larvae. Type I hepcidin was more abundantly expressed than Type II hepcidin and was also expressed at an earlier time (5 dph vs. 9 dph.).

Figure 20 describes Reverse transcription-PCR assay of hepcidin and actin gene expression in different tissues of winter flounder. Amplification products from adult 25 winter flounder were amplified using gene-specific primers for Flatfish Type I (panel A), Type II (panel B) and Type III (panel C) hepcidins and for actin (310 bp) and resolved by electrophoresis on a 2% agarose gel. Markers (M) are the 100 bp ladder (BRL)

30 Figure 21 describes Reverse transcription-PCR assay of hepcidin and actin gene expression in different tissues of control Atlantic salmon (C) and those infected with *Aeromonas salmonicida* (I). Amplification products from reactions using gene-specific primers for Salmonid Type I (panel A) and Type II (panel B) hepcidins (163

bp) and for actin (400 bp) were resolved by electrophoresis on a 2% agarose gel. Markers (M) are the 100 bp ladder (BRL).

Figure 22 describes Reverse transcription-PCR assay of hepcidin and actin expression in developing winter flounder larvae. Samples were larvae at 5 dph (lane 1), 12 dph (lane 2), 19 dph (lane 3), 27 dph (lane 4), 41 dph (lane 5) and adult (lane 6). Amplification products from reactions using gene-specific primers for Flatfish Type I (panel A), Type II (panel B) and Type III (panel C) hepcidins and for actin (400 bp) were resolved by electrophoresis on a 2% agarose gel using a 100 bp ladder (Pharmacia) as markers (lane M).

Identification of additional hepcidin-like sequences from other fish species

Using a primer based on highly conserved sequences in the signal peptide of all reported hepcidins (Hep Universal 5') in combination with primers based on highly conserved sequences in the 3' UTR of salmon (HcSal 3') and flatfish (HcPA3b 3'), it was possible to amplify hepcidin-like sequences from the liver and spleen of halibut and salmon (Fig. 2). An alignment of the deduced amino acid sequences of hepcidin-like peptides from winter flounder, Atlantic halibut and Atlantic salmon is shown in Fig. 17. Interestingly, flatfish-type hepcidin could be amplified from salmon (S8.6) and salmon-type hepcidin could also be amplified from a flatfish (Hb7.5). Additional sequences were obtained from genomic DNA of Petrale sole, C-O sole, English sole, starry flounder, European plaice, Greenland halibut and Pacific halibut.

Figure 17 depicts an alignment of certain winter flounder (WF1, WF2, WF3a, WF3b, WF4) Atlantic halibut (Hb1.1, Hb5.3, Hb7.5, Hb17, Hb357) and Atlantic salmon (Sal1, Sal2, Sal2.1, Sal8.6) hepcidins with those of Japanese flounder (JFL4, JFL6, medaka, hybrid striped bass and human. A partial sequence from rainbow trout (Genbank Accession AF281354_1) is also shown. The predicted positions of signal peptidase and pre-protein cleavages are indicated by arrows.

DISCUSSION

Pleurocidins

Most antimicrobial peptides, including cecropins and dermaseptins, are encoded by multigene families that have probably arisen by sequential gene duplications. We have demonstrated that the winter flounder, and probably other flatfish, possess a gene family encoding antimicrobial compounds similar to pleurocidin. Comparison of the genomic amplification products obtained using PL1/2 with the cDNA sequence (Fig. 1A) showed that WF2 and WF4 contain three introns, the first of which occurs only 1 bp upstream from the initiator methionine. The second and third introns both occur within the mature peptide. The genes for GLa, xenopsin, levitide and caerulein – all skin peptides from *Xenopus laevis* – also contain an intron 1 bp upstream from the initiator methionine (Kuchler et al 1989). The intron positions are conserved in all but WF3 (Fig. 6), but they differ dramatically in size (Table 5), indicating that a considerable period of evolutionary time has elapsed since the duplication events occurred, or that the intron sequences are relatively free to drift.

Southern analysis shows that WF1-4 probes hybridise to other flatfish DNAs, including yellowtail flounder, Atlantic halibut and American plaice, but not to haddock, smelt or pollock. This hybridisation could be due to the highly conserved signal sequence and anionic portion which we have shown to be conserved in sequences isolated from these flatfish. Flatfish may provide a rich reservoir of potential therapeutants for the aquaculture industry. The probes for the different pleurocidin family members often recognise the same restriction fragments in winter flounder DNA, indicating that they may be clustered at a single locus on the genome. Complete sequencing of two Lambda clones hybridizing to pleurocidin confirms that such clustering does in fact occur (Fig. 11). Clustering of antimicrobial peptide genes has also been noted for insect cecropins (Gudmundson et al. 1991) and apidaecins (Casteels-Jossen et al. 1993), among others.

Figure 11 describes an embodiment of a Schematic of genomic organization of pleurocidin-like genes and pseudogenes (ψ) from winter flounder. Introns are represented by solid boxes and exons by stippled boxes.

All of the members of the pleurocidin family are encoded as prepolypeptides consisting of an amino-terminal signal sequence followed by the active peptide and ending with an acidic portion. The deduced amino acid sequences of the signal and acidic sequences are very highly conserved whereas those of the predicted mature antimicrobial peptides are more variable (Fig. 6). All, however, appear to fold into amphipathic α -helices. This sequence conservation has allowed us to use a genomic approach to identify many different members of the pleurocidin gene family, not only from winter flounder but also from a variety of other flatfish (Fig. 3, Table 4, Appendix I).

The structure of the pleurocidin prepro polypeptides bears certain resemblances to the frog dermaseptin precursors, which also contain a signal sequence of similar length (22 amino acids) and an acidic portion of 16-25 amino acids. From the full-length cDNA clone (Fig. 1A), the acidic portion of pleurocidin was shown to contain 21 residues. A major difference between the pleurocidin and dermaseptin prepolypeptides is the position of the acidic portion – downstream of the mature peptide in pleurocidin and upstream of the mature peptide in dermaseptins. The acidic propeptides of defensins have been proposed to prevent interaction of the antimicrobial peptide with the membrane by neutralising the cationic charges (Valore et al. 1996) and this may also be its function in pleurocidin. This feature can be of practical significance for delivering peptides that are inactive until specifically cleaved.

The signal sequences and acidic carboxy-terminal sequences of the pleurocidin family members are extremely highly conserved. The former, and possibly the latter, are presumed to target the precursor molecules to the cell membrane for secretion. Gene families for antimicrobial peptides that contain highly conserved signal peptides (often encoded by the first exon) followed by end products with different biological activities have been described from the dermaseptin family (Valore et al. 1996) and the GLa, xenopsin, lexitide and caerulein, all of which are skin peptides from *Xenopus laevis* (Kuchler et al. 1989). These authors proposed that this modular gene structure allows targeting for secretion to be achieved for markedly different peptides using a common pathway. In the pleurocidin gene family, a modular structure is also present with exon 2 encoding the signal sequence and first

half of the antimicrobial peptide, exon 3 encoding the next ten amino acids of the antimicrobial peptide, and exon 4 encoding the last three amino acids of the antimicrobial peptide and the acidic carboxy terminus.

5 The mature peptides encoded by WF2 and WF4 are 60% identical to each other (Fig. 6) and somewhat less similar to dermaseptin B1 and ceratotoxin B (Cole et al. 1997). WF1 is 64% identical to WF1a but contains a remarkably cationic stretch of 18 amino acids between the signal sequence and the mature peptide that is not present in WF1a. Whether or not this potentially antimicrobial 18-mer peptide arises when
10 pleurocidin WF1 processing occurs remains to be determined. Both WF1 and WF1a contain an additional 10-11 amino acids relative WF2, WF3 and WF4 between the mature peptide and the acidic carboxy terminus. WF3 shares similarities with both WF2/4 and WF1/1a. Synthetic pleurocidin identical to the central portion of WF2 has been shown to protect Coho salmon against infection by *Vibrio anguillarum*, as have
15 hybrid peptides based on pleurocidin, dermaseptin and ceratotoxin (Jia et al. 2000).

 The tissue-specific expression of the pleurocidin genes was assessed using northern blot analysis and RT-PCR. Northern analysis proved to be not sufficiently sensitive for detecting the low level of transcripts present in winter flounder mRNA. Transcripts were present only in skin in sufficient quantities to be detected by this
20 method, so the more sensitive RT-PCR assay was used. Pleurocidin transcripts were found in both skin and intestine using this method, in agreement with the recently reported ultrastructural localisation of pleurocidin in these tissues (Cole, Darouiche et al. 2000) and supporting the role of pleurocidin in mucosal immunity. The transcript size (approximately 200 bp) is consistent with the size of products obtained by RT-
25 PCR (Table 7), showing that the pleurocidin genes are transcribed separately.

 RT-PCR analysis showed that the genes for the different pleurocidin-like peptides are expressed in a tissue-specific manner with WF2 being expressed predominantly in the skin and gill and to a lesser extent in the muscle, intestine,
30 stomach and liver whereas WF1 and WF4 are detected predominantly in the gill and skin (Fig. 7). WF3 and WFYT are expressed in most of the tissues sampled, WFX is detected solely in the skin and WF1a was not expressed in any of the tissues sampled. Possibly, the different antimicrobial peptides are required to control the growth of different bacterial populations in the two tissues. Since no RT-PCR products were

detected for WF4, it is possible that this gene is expressed only at low levels in adult skin or intestine or that it is expressed at a different life stage or in a different tissue.

Using primers that did not discriminate between the transcripts of the various pleurocidin-like genes, expression was first detected at 5 dph and showed a progressive increase towards adulthood. However, recent experiments using primers specific for WF1, WF1a, WF2, WF3, WF4, WFX and WFYT, transcripts were detected at different developmental stages (Fig. 9). WFX was only detectable at 20 dph, whereas WFYT, WF3 and WF2 were detectable at 5 dph and at higher levels between 25-36 dph. Interestingly, WF1 was not detectable at any larval stage and may only be expressed under specific environmental conditions in response to specific bacterial pathogens, as has been shown for *Drosophila* (Rivas and Ganz 1999). This is the first demonstration of developmental expression of an antimicrobial peptide in fish and shows that at least this component of innate immunity is present in early larval stages of winter flounder. Larval mortality prior to metamorphosis is of great concern and although the reasons for such mortality are not yet known, high bacterial load in the gut has been proposed (Padros, Minkoff et al. 1993). The adaptive immune systems of flatfish have been shown to develop later than those of other teleosts (Padros, Sala et al. 1991). Thus, the ability of larvae to produce antimicrobial peptides during this period may be crucial to survival, and the identification of factors that increase the production of such compounds would be of great benefit to aquaculturalists.

These results of testing synthetic peptides against a variety of bacterial pathogens as well as the fungal pathogen, *Candida albicans*, show promising candidates with broad-spectrum antimicrobial activities. Of particular interest is the ability of the peptides NRC-13 and NRC-15 to inhibit the growth of methicillin-resistant *S. aureus* at concentrations as low as 4 µg/ml. NRC-13 is also capable of inhibiting the growth of *C. albicans* at 4 µg/ml, *P. aeruginosa* at 1 µg/ml (and killing *P. aeruginosa* at this concentration), and *A. salmonicida* at 2 µg/ml. This means that NRC-13 is highly active against a fish pathogen, a Gram-negative human bacterium, a drug-resistant Gram-positive human bacterium, and a yeast. The example of NRC-13 demonstrates the range of potential targets and applications for cationic antimicrobial peptides.

These results also validate the process we used for selecting antimicrobially active peptides from a large amount of sequence data. The ability to accurately predict which peptides are likely to be active is a crucial link between genomics and therapeutics. While much work remains to be done in this area, we have clearly demonstrated that judicious application of the principles described earlier will aid in selecting active peptides.

Thus, a variety of cDNA and genomic sequences encoding the precursors of antimicrobial peptides identical to or similar to pleurocidin from a variety of flatfish species have been isolated. Northern hybridisation and sequence analysis of RT-PCR products showed that expression was tissue-specific. Most importantly, the timing of expression of different pleurocidin variants in developing larval winter flounder was determined, allowing an estimate of the onset of the innate immune system in this fish. These assays of pleurocidin expression are useful in directing the screening strategy for isolating novel peptide sequences expressed during specific tissues and/or developmental stages. Environmental parameters affecting the production of pleurocidin can also be assayed.

This work paves the way to further studies aimed at the over-expression of pleurocidin as a therapeutant for aquacultured fish and the production of disease-resistant fish through transgenic technology as has been demonstrated in transgenic tobacco expressing antimicrobial peptides (Jach et al. 1995) and proposed for fish (Jia et al. 2000). Furthermore, because many fish live in a saline environment, the properties of their antimicrobial peptides may be different from those produced by terrestrial animals and have application in unique situations. For instance, the pulmonary mucosa of patients with cystic fibrosis contain elevated NaCl concentrations, which inhibit the natural cationic peptides secreted by the lung (Goldman et al. 1997). Salt-adapted cationic peptides from marine fish may have application in the treatment of lung infections in these patients.

Hepcidins

Sequence analysis of one salmon EST (SL1-0412) and one halibut clone (Hb7.5), revealed the presence of unspliced transcripts and allowed the positions of some of the introns to be determined (Fig. 16). Similar to mouse, human and hybrid striped bass, the salmon hepcidin is composed of three exons and two introns (Park, Valore et al. 2001; Shike et al. 2002; Pigeon, Ilyin et al. 2001). The position of the first intron of salmon and bass are identical and correspond to a position two amino acids 5' to those of mouse and human. However, the second salmon intron and the second halibut intron of Hb7.5 correspond to a position two amino acids 3' to those of mouse and human and several amino acids 5' to that of the bass. This is probably due to "intron sliding" whereby the positions of introns have shifted by several nucleotides over the course of evolution. Interestingly, the deletion in WF4 corresponds precisely to the position of the first salmon intron and the second mouse/human intron, indicating an intermediate intron/exon structure.

Mouse contains two hepcidin genes that are clustered on the genome (Pigeon, Ilyin et al. 2001) but in human (Park, Valore et al. 2001) and striped bass (Shike et al. 2002) only one hepcidin gene has been identified. Although the number of hepcidin genes in winter flounder and Atlantic salmon remains to be determined, there are at least five in winter flounder, five in Atlantic halibut and four in Atlantic salmon. Since there are no *Sst*I sites within the hepcidin probe used in the Southern hybridization analysis, it is highly probable that the five winter flounder hepcidin genes reported here are clustered on two genomic fragments. Multiple genes for pleurocidin also exist (Douglas, Gallant et al. 2001) and are clustered on the genome (Fig. 11). Interestingly, all of the small flounders tested from the Atlantic exhibited a similar hybridizing band of 4.3 kb, indicating that they share similarity at the genomic level. Japanese flounder, found in the Pacific, exhibited a single hybridizing band of 5.5 kb.

The deduced amino acid sequences of the fish prepro-hepcidins can be aligned with those from mammals throughout their length but only show high similarity in the portion corresponding to the processed peptides (Fig. 17). However, within the fish, the signal peptide and the propiece are also very highly conserved. Conservation of

these segments has also been noted in the pleurocidin family (Douglas, Gallant et al. 2001). The amino-termini of the processed peptides were assigned based on the amino acid sequence of human hepcidin (Krause, Neitz et al. 2000; Park, Valore et al. 2001) and the proximity to the RX(K/R)R motif characteristic of processing sites
5 (Nakayama 1997). The molecular weights of the processed hepcidins from winter flounder and Atlantic salmon range from 1992 Da (WF2) to 3066 (WF1), comparable to hepcidins isolated from mouse, human and bass. With the exception of WF2, which has an acidic pI (5.54), the pIs of hepcidins are between 7.73 and 8.76.

10 Like pleurocidins, the amino acid sequences of the hepcidin variants are highly similar within species, suggesting relatively recent duplication of an ancestral gene. It is possible that the aquatic environment in which fish live necessitates the existence of a more diverse suite of antimicrobial peptides than in terrestrial mammals. In addition, this component of the innate immune system plays a more
15 major role in fish than in mammals, which have a more highly evolved adaptive immune system.

The human hepcidin molecule has been proposed to form a secondary structure containing a series of β -turns, loops and distorted β -sheets (Park, Valore et al. 2001). Consensus secondary structure prediction of fish hepcidins show that they
20 contain mostly random coil structure with some extended strand structure. With the exception of WF2, JFL6 and Hb357, all hepcidins reported thus far contain eight cysteine residues which are proposed to form four disulphide bonds (Krause, Neitz et al. 2000; Park, Valore et al. 2001) in the following linkage pattern: 1-4, 2-8, 3-7, 5-6
25 (Park, Valore et al. 2001). The loss of cysteine residues 1 and 3 from WF2 suggests that at least one disulphide bond cannot form.

Using gene-specific primers, we were able to demonstrate that different hepcidin genes are expressed in different tissues of both winter flounder (Fig. 20) and
30 Atlantic salmon (Fig. 21). In Atlantic salmon, hepcidin was detectable in normal uninfected fish predominantly in liver, blood and muscle (Type I) and to a lesser extent in gill and skin (both types). This is consistent with the presence of three ESTs for Type I hepcidin in cDNA libraries constructed from uninfected livers, and the absence of ESTs for Type II hepcidin in cDNA libraries constructed from uninfected

liver, spleen and head kidney. Type II hepcidin expression appears be confined to external epithelial surfaces in contact with the aqueous environment, whereas Type I hepcidin expression is more widespread, being expressed in liver, blood and muscle as well as external epithelial surfaces. In uninfected winter flounder, no transcripts of Type II hepcidin could be detected in any tissue but transcripts of Types I and III hepcidin were present in the liver and cardiac stomach. Type III hepcidin transcripts were also present in the esophagus.

Mouse hepcidin was also reported to be predominantly expressed in liver, and weakly in stomach, intestine, colon, lungs, heart and thymus by Northern analysis using one of the mouse hepcidin sequences as probe (Pigeon, Ilyin et al. 2001). However, this study did not discriminate between the two hepcidin genes and it is not known whether or not the two mouse genes are differentially expressed in tissues of mouse. Similarly, dot-blot analysis of human tissues and cell lines using the human hepcidin cDNA as probe revealed strong expression in adult and fetal liver and weaker expression in adult heart, fetal heart and adult spinal cord (Pigeon, Ilyin et al. 2001). An earlier study using RealTime quantitative RT-PCR (Krause, Neitz et al. 2000) revealed strong expression of hepcidin in human liver, heart and brain and weak expression in a variety of other tissues. Interestingly, we could not detect either Type I or Type II hepcidin expression in the brain of normal Atlantic salmon or winter flounder, or heart of normal Atlantic salmon. However, in infected animals, Type II hepcidin was expressed in both tissues, indicating that this form is the predominant one produced under conditions of stress.

It is intriguing that we detected transcripts of Type I hepcidin that were constitutively expressed in blood cells of Atlantic salmon. Constitutively expressed non-enzymic antimicrobial molecules have been reported only rarely in blood of fish; a small hydrophobic cationic peptide was found in mucus of rainbow trout (Smith et al., 2000) and moronecidin, an antimicrobial peptide from bass, was expressed in blood of uninfected animals (Lauth et al. 2002). Interestingly, expression of neither hepcidin increased in blood of infected salmon relative to the uninfected control animals. Possibly, hepcidin is fulfilling a role in iron homeostasis in control animals as well as an antimicrobial role. Its presence in circulating blood cells of uninfected animals may be a precautionary measure against impending infection.

Type I and II hepcidins from Atlantic salmon were up-regulated during infection with *Aeromonas salmonicida*, but to different extents in various tissues. While Type I hepcidin was noticeably up-regulated in the esophagus, stomach, pyloric caecae, liver, spleen, intestine, posterior kidney, rectum and muscle and to a lesser extent in anterior kidney and skin, Type II hepcidin showed a more dramatic increase in stomach, pyloric caecae, liver, spleen, intestine, brain, heart and muscle. Weaker up-regulation was present in esophagus, anterior and posterior kidney, skin and rectum. These results are consistent with those reported for bacterially challenged hybrid striped bass where up-regulation was most dramatic in liver, but was also demonstrated in skin, gill, intestine, spleen, anterior kidney and blood (Shike et al. 2002). It is not known whether there are multiple hepcidins in hybrid striped bass and, if so, whether they are differentially expressed as in Atlantic salmon and winter flounder.

Studies with mice have shown a 4.3-fold increase in hepcidin expression in livers of mice injected with LPS and a 7-fold increase in primary hepatocytes exposed to LPS (Pigeon, Ilyin et al. 2001). These studies were based on Northern analysis using only one of the mouse hepcidin sequences as probe, and were therefore unable to distinguish possible differential expression of the two mouse variants. Similar increases were noted in livers of mice subjected to iron overload, but not for primary hepatocytes exposed to iron citrate, possibly due to the differentiation status of the cultured hepatocytes. The fact that both iron overload and LPS exposure increase hepcidin expression indicates the importance of these two factors in the host response to pathogens.

During infection, iron is removed from the system by various mechanisms so that it is unavailable for use by invading pathogens. It has been proposed that recently discovered transferrin receptor2 mediates iron uptake by hepatocytes and increases their expression of hepcidin (Fleming and Sly 2001; Nicolas, Bennoun et al. 2001). Hepcidin, in turn, increases iron accumulation in macrophages and increases dietary iron absorption in duodenal crypt cells via $\beta 2$ microglobulin, HFE and transferrin receptor1. These crypt cells differentiate into enterocytes with reduced amounts of iron transport proteins, thereby decreasing dietary iron uptake. Hepcidin thus appears to play a crucial role in iron homeostasis during inflammation as well as acting as an

antimicrobial peptide. It is also possible that hepcidin could modulate expression of liver-derived acute phase proteins and exhibit synergistic effects with other components of the immune system.

5 Antimicrobial peptides have been shown to modulate gene expression in mouse macrophages (Scott, Rosenberger et al. 2000), and it is possible that they may exert similar effects in fish macrophages or hepatocytes. The presence of a functional nuclear localization signal (four K/R residues in a row) within prohepcidin of mouse and human indicates that hepcidin could act as a signaling molecule involved in
10 maintenance of iron homeostasis in these organisms (Pigeon, Ilyin et al. 2001). Interestingly, the nuclear localization signal also contains the recognition signal for processing of prohepcidin, indicating that nuclear localization would occur only prior to removal of the propiece, or that the propiece itself is localized to the nucleus. Teleost hepcidins contain only 3 out of 4 K/R residues, which may not be sufficient
15 for nuclear localization; a role for hepcidin in intracellular signaling awaits testing with synthetic or *in vitro*-expressed peptide.

 In conclusion, the sequences of new hepcidin-like peptides from different fish species and the presence of related sequences in several flatfish species by Southern
20 hybridization have been determined. Furthermore, it has been shown that the various types of fish hepcidins are differentially expressed in a tissue-specific manner in normal fish, as a result of bacterial infection, and during larval development, thus providing a strategy for identifying additional sequences for novel peptides. Apparently in fish, different tissues produce hepcidins in a constitutive or inducible
25 manner, indicating that hepcidin variants may have different functions under different circumstances. Given their role in iron homeostasis in mammals, it is possible that fish hepcidin variants may fulfill this role as well as that of killing specific pathogens. *In vitro* expression of hepcidin variants will allow their spectrum of antimicrobial activity to be determined as well as their effect on the innate immune response.

30 Thus, there has been provided a method for identifying potential antimicrobial peptides.

Tables

- Table 1. Nucleotide sequences of oligonucleotides used for isolating pleurocidin-like sequences
- 5 Table 2. Nucleotide sequences of oligonucleotides used for assay of pleurocidin-like gene expression in different tissues and at different stages of development of winter flounder
- Table 3. Nucleotide sequences of primers used in RT-PCR assays to analyse hepcidin gene expression. The amino acid sequence on which the 5' primer was based is
- 10 shown. The 3' primers were within the 3' untranslated region (3' UTR). The annealing temperatures used in the PCR reactions and the sizes of the amplification products are listed.
- Table 4. One-letter amino acid sequences for pleurocidins based on genomic and expression data
- 15 Table 4a. Bacterial and *Candida* strains used in this study
- Table 5. Sizes of introns (in bp) in genomic sequences amplified using primers PL5' and PL3'
- Table 6. RT-PCR products from skin and intestine corresponding to different pleurocidin genes
- 20 Table 7. Sizes of bands (in kb) hybridising to pleurocidin probes in *Bam*HI and *Sst*I digests of winter flounder DNA
- Table 8. Minimal inhibitory concentrations of pleurocidin-like cationic antimicrobial peptides against a wide spectrum of bacterial pathogens and *Candida albicans*.
- Table 9. Characteristics of winter flounder and Atlantic salmon hepcidin-like peptides
- 25 Table 10. Results of PCR analysis of hepcidin expression
- Table 11. One-letter amino acid sequences for certain hepcidins based on genomic and expression data, including NRC reference numbers
- Table 12. Nucleotide sequences corresponding to amino acid sequences listed in Tables 11 and 13
- 30 Table 13 One-letter amino acid sequences for certain hepcidins based on genomic and expression data, including clone names

Appendices

APPENDIX I. NUCLEOTIDE SEQUENCES OF PLEUROCIDIN-LIKE GENES AND CDNAS REFERRED TO IN TABLE 4.

Appendix II. Nucleotide sequences of hepcidin-like genes and cDNAs referred to in

5 Table 11.

References

The mention of a reference is not an admission or suggestion that it is relevant to the patentability of anything disclosed herein.

Amsterdam, D. 1996. Susceptibility Testing of Antimicrobials in Liquid Media. *In* V.
10 Lorian (ed.), Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore.

Casteels-Jossen, K., T. Capaci, et al. (1993). "Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response." EMBO J. 12: 1569-78.

15 Cohen, S., M. Skiguchi, J. Stern, and H. Barner. 1963. The synthesis of messenger RNA without protein synthesis in normal and phage-infected thymineless strains of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A Biochem. 49:699-706.

20 Cole, A. M., R. O. Darouiche, et al. (2000). "Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity." Antimic. Ag Chemotherapy. 44: 2039-45.

Cole, A. M., P. Weis, et al. (1997). "Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder." J. Biol. Chem. 272(18): 12008-12013.

25 Douglas, S. E., C. E. Bullerwell, et al. (1999). "Molecular investigation of aminopeptidase N expression in the winter flounder, *Pleuronectes americanus*." J. Appl. Ichthyol. 15: 80-86.

Douglas, S. E., J. W. Gallant, et al. (1999). "Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes." Mar. Biotechnol. 1: 458-464.

- Douglas, S. E., J. W. Gallant, et al. (1998). "Isolation of cDNAs for trypsinogen from the winter flounder, *Pleuronectes americanus*." J. Mar. Biotechnol. **6**: 214-9.
- Douglas, S. E., J. W. Gallant, et al. (2001). "Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum)." Dev. Comp. Immunol. **25**: 137-147.
- Douglas, S. E., A. Gawlicka, et al. (1999). "Ontogeny of the stomach in winter flounder: characterisation and expression of the pepsinogen and proton pump genes and determination of pepsin activity." J. Fish Biol. **55**: 897-915.
- Douglas, S. E., S. C. M. Tsoi, et al. (2002). Expressed sequence tags - a snapshot of the fish genome. A Step Toward the Great Future of Aquatic Genomics, Tokyo, Japan.
- Fleming, R. E. and W. S. Sly (2001). "Hepcidin: A putative iron-regulatory hormone relevant to hereditary hemochromatosis and the anemia of chronic disease." Proc. Natl. Acad. Sci. USA **98**(15): 8160-8162.
- Garvan, J. (1996). SeqVu. Sydney, Australia, The Garvan Institute of Medical Research.
- Goldman, M. J., G. M. Anderson, et al. (1997). "Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis." Cell. **88**: 553-60.
- Gong, Z., K. V. Ewart, et al. (1996). "Skin antifreeze protein genes of the winter flounder, *Pleuronectes americanus*, encode distinct and active polypeptides without the secretory signal and prosequences." J. Biol. Chem. **271**: 4106-12.
- Gudmundsson, G. H., D. A. Lidholm, et al. (1991). "The cecropin locus. Cloning and expression of a gene cluster encoding three antibacterial peptides in *Hyalophora cecropia*." J. Biol. Chem. **166**: 11510-7.
- Hwang, E.-Y., J.-K. Seo, et al. (1999). "Purification and characterization of a novel antimicrobial peptide from the skin of the hagfish, *Eptatretus burgeri*." J. Food Sci. Nutr. **4**(1): 28-32.

- Jach, G., B. Gornhardt, et al. (1995). "Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco." Plant J. **8**: 97-109.
- 5 Jia, X., A. Patrzykat, et al. (2000). "Antimicrobial peptides protect coho salmon from *Vibria anguillarum* infections." Appl. Environ. Microbiol. **66**: 1928-32.
- Krause, A., S. Neitz, et al. (2000). "LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity." FEBS Lett. **480**: 147-150.
- 10 Kuchler, K., G. Kreil, et al. (1989). "The genes for the frog skin peptides GLAa, xexopsin, levitide, and caerulin contain a homologous export exon encoding a signal sequence and part of an amphiphilic peptide." Eur. J. Biochem. **179**: 281-5.
- Lauth, X., H. Shike, et al. (2002). "Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass." J. Biol. Chem. **277**: 5030-5039.
- 15 LeMaitre, C., N. Orange, et al. (1996). "Characterization and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*)." Eur. J. Biochem. **240**: 143-149.
- Marck, C. (1992). DNA Strider Version 1.2. Service de Biochimie - Bat 142, Centre d'Etudes Nucleaires de Saclay, Gif-sur-Yvette, France.
- 20 Moore, K. S., S. Wehrli, et al. (1993). "Squalamine: an aminosterol antibiotic from the shark." Proc. Natl. Acad. Sci. USA. **90**: 1354-1358.
- Nakayama, K. (1997). "Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins." Biochemical J. **327**: 625-635.
- 25 Nicolas, G., M. Bennoun, et al. (2001). "Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice." Proc. Natl. Acad. Sci. USA. **98**(15): 8780-8785.

- Oren, Z. and Y. Shai (1996). "A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from Moses sole fish *Pardachirus marmoratus*." Eur. J. Biochem. **237**(1): 303-310.
- 5 Padros, F., G. Minkoff, et al. (1993). "Histopathological events throughout the development of turbot (*Scophthalmus maximus* L.)." J. Comp. Pathol. **109**: 321-4.
- Padros, F., R. Sala, et al. (1991). Organogenesis in turbot, *Scophthalmus maximus*, larvae related to the main developmental stages: in Larvi'91. Fish and Crustacean Larviculture Symposium. Ghent, Belgium: European Aquaculture Society.
- 10 Park, C. B., J. H. Lee, et al. (1997). "A novel antimicrobial peptide from the loach, *Misgurnus anguillicandatus*." FEBS Lett. **411**: 173-178.
- Park, C. H., E. V. Valore, et al. (2001). "Hepcidin, a urinary antimicrobial peptide synthesized in the liver." J. Biol. Chem. **276**(11): 7806-7810.
- Park, I. Y., C. B. Park, et al. (1998). "Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*." FEBS Lett. **437**(3): 258-262.
- 15 Pigeon, C., G. Ilyin, et al. (2001). "A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload." J. Biol. Chem. **276**(11): 7811-7819.
- Rivas, L. and T. Ganz. (1999). "Eukaryotic antibiotic peptides: not only a membrane business." Drug Discovery Today. **4**: 254-6.
- 20 Scott, M. G., C. M. Rosenberger, et al. (2000). "An α -helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression." J. Immunol. **165**: 3358-3365.
- Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Shimizu C, Burns JC (2002). "Bass hepcidin is a novel antimicrobial peptide induced by
25 bacterial challenge." Eur J Biochem : **269**:2232-2237.
- Silphaduang, U. and E. J. Noga (2001). "Peptide antibiotics in mast cells of fish." Nature **414**: 268-9.

Smith, V. J., J. M. O. Fernandes, et al. (2000). "Antibacterial proteins in rainbow trout, *Oncorhynchus mykiss*." Fish Shellfish Immunol. **10**: 243-260.

5 Thompson, J., D. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice." Nucleic Acids Res. **22**: 4673-4680.

Trust T. J., Ishiguro, E. E., Chart, H. and Kay W. W. (1983) Virulence properties of *Aeromonas salmonicida*. J. World Maricul. Soc. **14**:193-200.

10 Valore, E. V., E. Martin, et al. (1996). "Intramolecular inhibition of human defensin HNP-1 by its propiece." J. Clin. Invest. **97**: 1624-9.

Wu, M., E. Maier, R. Benz, and R. E. W. Hancock. 1999. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochem. **38**:7235-7242.

Table 1. Nucleotide sequences of oligonucleotides used for isolating pleurocidin-like sequences

Primer	Amino Acid Sequence	Nucleotide Sequence (5' \Rightarrow 3')
<i>Screening cDNA library</i>		
PleuroA	FFKKAHVGH	TTCTTCAAGAAGGCGCYCAYGT[C/G]GG [C/A]AAGCA
PleuroB	HVGKAALTHYL ¹	CAYGT[C/G]GG[C/A]AAGGCGCYCT[C/G] AA[C/T/A]CAYTACCT
<i>Genomic PCR and RT-PCR</i>		
PL1	5' untranslated	GCCCACTTTGTATTCGCAAG
PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
PL5'	MKFTATF	ATGAAGTTCACTGCCACCTTC
PL3'	KRAVDE ¹	TCATCGACTGCGCGCTT
¹ complement		

Table 2. Nucleotide sequences of oligonucleotides used for assay of pleurocidin-like gene expression in different tissues and at different stages of development of winter flounder

Gene	Primer	Amino Acid Sequence	Nucleotide Sequence (5' \Rightarrow 3')
WF1	RTWF1	KGRWLER	AAGGGCAGGTGGTTGGAAAGG
	RTWF1/3'	YQEGEE ¹	CCCTCCCCCTCCTGGTA
WF1a	RTWF1a	RKRKWLR	CGTAAGAGAAAGTGGTTGAGA
	RTWF1a/3'	YQEGEE ¹	CCCTCCCCCTCCTGGTA
WF2	RTWF2	KAAHVG	AAGGCTGCTCACGTTGGC
	PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
WF3	RTWF3	FLGALIK	TTCTTAGGAGCCCTTATCAAA
	RTWF3/3'	YDEQQE ¹	CTCCTGCTGCTCGTCATA
WF4	RTWF4	HGRHAA	CATGGTCGTCATGCTGCC
	PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
WFYT	RTWFYT	GFLFHG	GGGATTTCCTTTTTCATGG
	RTWFYT/3'	SFDDNP ¹	GGGTTGTCATCGAATGAG
WFX	RTWFX	RSTEDI	CGTTCTACAGAGGACATC
	RTWFX/3'	DDDDSP ¹	GGGGCTGTCATCATCATC

Table 3. Nucleotide sequences of primers used in RT-PCR assays to analyse hepcidin gene expression. The amino acid sequence on which the 5' primer was based is shown. The 3' primers were within the 3' untranslated region (3' UTR). The annealing temperatures used in the PCR reactions and the sizes of the amplification products are listed.

Type	Primer Product	Amino acid	Nucleotide sequence	Annealing	
(size)		sequence	(5' \Rightarrow 3')	temperature	size
(bp)					
<i>Winter flounder</i>					
Type I	HcPA1 5' 137	WMENPT	TGGATGGAGAATCCCACC	50°C	
	HcPA1b 3'	3' UTR	GTGAGGTTGTGTTGCGGG		
Type II	HcPA2 5' 180	GMMPNN	GGGATGATGCCAAACAAC	50°C	
	HcPA2b 3'	3' UTR	ACTTGGACTATGGGCTGAG		
Type III	HcPA3 5' 118	WMMPNN	TGGATGATGCCATACAAC	50°C	
	HcPA3b 3'	3' UTR	GTTGTTGGAGCAGGAATCC		
Actin	ActF (WF) 312	AALVVD	TCGCTGCCCTCGTTGTTGAC	50°C	
	ActR (WF)*	VLLTEAP*	GGAGCCTCGGTCAGCAGGA		
	ActinF1 194	VFPSIV	GTGTTCCATCCATCGTC	50°C	
	Actin R1	HTFYNEL	GAGCTCGTTGTAGAAGGTGT		
<i>Atlantic salmon</i>					
Type I	HCSS 5' 163	MHLPEP	ATGCATCTGCCGGAGCCT	55°C	
	Hep Liv R	3' UTR	CATTGCAAACATGTACAAACTAG		
Type II	Hep Sp F 163	MNLPMH	ATGAATCTGCCGATGCA	52°C	
	Hep Sp R	3' UTR	GGGCAAATTAAAGGCG		
Actin	Act400F 400	IVGRPRHQ	TCGTGGTTCGTCCCAGGCATCAG	52°C	
	Act400R	GYALPHAI	ATGGCGTGGGGCAGAGCGTAACC		

* complement

Table 4. Sequences of pleurocidin-like peptides used for activity testing. Final peptide sequences and patterns of C-terminal amidation were selected based on the analysis of translated nucleotide sequences and on principles described in the text.

Origin	Amino acid sequence	Code
Winter Flounder (1)	GKGRWLERIGKAGIIGGALDHL-NH ₂	NRC-01 ^a
Winter Flounder (1a)	WLRRIGKGVKIIGGAALDHL-NH ₂	NRC-02 ^{a, d}
Winter Flounder (1a-l)	GRRKRKWLRRIGKGVKIIGGAALDHL-NH ₂	NRC-03 ^{a, d}
Winter Flounder (2) 2.1	GWGSFFKKAHVGVKGVKAALTYL-NH ₂	NRC-04 ^a
Winter Flounder (3)	FLGALIKGAIHGRFTHGMIQNHH-NH ₂	NRC-05 ^a
Winter Flounder (4) 1.1	GWGSIFKHGRHAAKHIGHAAVNHYL-NH ₂	NRC-06 ^a
Yellowtail Flounder YT2	RWGKWFKKATHVGVKGVKAALTYL-NH ₂	NRC-07 ^b
Winter Flounder X	RSTEDIKISISGGFNLAMNA-NH ₂	NRC-08 ^{b, c}
Winter Flounder Y	FFRLLFHGVHGGGYLNAA-NH ₂	NRC-09 ^{b, c}
Winter Flounder Z	FFRLLFHGVHGVKIKPRA-NH ₂	NRC-10 ^{b, c}
American Plaice AP1	GWKSVERKAKVKTGGGLADHYL-NH ₂	NRC-11 ^b
American Plaice AP2	GWKKWFNRAKVKTVGGGLAVDHYL-NH ₂	NRC-12 ^b
American Plaice AP3	GWRTLKKAETVTKLALKHLYL-NH ₂	NRC-13 ^b
Witch Flounder GcSc4C5	AGWGSIFKHIFFKAGFIHGAIQAHD-NH ₂	NRC-14 ^b
Witch Flounder GcSc4B7	GFWGKLFKGLHGIGLLHLHL-NH ₂	NRC-15 ^b
Witch Flounder GC3.8-t	GWKKWLRKGAKHLGQAAIK-NH ₂	NRC-16 ^b
Witch Flounder GC3.8	GWKKWLRKGAKHLGQAAIKGLAS	NRC-17 ^b
Witch Flounder GC3.2	GWKKWFTKGERLLSQRHFA	NRC-18 ^b
Halibut Hb26	FLGLLFHGVHGVKWIHGLIHGHH-NH ₂	NRC-19 ^b
Halibut Hb18	GFLGLFHGVHGHGRKKALHMNSERRS	NRC-20 ^b

^a Peptide predicted from expressed tag and/or expression confirmed by RT-PCR and/or by *in situ* hybridization.

^b Peptide predicted from genomic sequence

^c Pseudogenes

^d NRC-2 and NRC-3 are both derived from the same sequences with the latter including an additional N-terminal fragment.

Table 4a. Bacterial and *Candida* strains used in this study.

Species	Code ID	Comments
<i>Escherichia coli</i>	C498, UB1005	Parent of DC2
<i>Escherichia coli</i>	C500, DC2	Outer membrane-permeable mutant
<i>Escherichia coli</i>	C786, CGSC4908	Triple auxotroph (thy, uri, L-his)
<i>Salmonella enterica</i> s. Typhimurium	C587, 14028S	Parent of C610
<i>Salmonella enterica</i> s. Typhimurium	C610, MS4252S	Supersusceptible strain
<i>Pseudomonas aeruginosa</i>	H187, K799	Parent of H188
<i>Pseudomonas aeruginosa</i>	H188, Z61	Supersusceptible strain
<i>Enterococcus faecalis</i>	C625, ATCC29212	Standard strain (ATCC)
<i>Staphylococcus aureus</i>	C622, ATCC25923	Standard strain (ATCC)
<i>Staphylococcus aureus</i>	C623, SAP017	<u>MRSA</u> clinical isolate (from Tony Chow – VGH)
<i>Staphylococcus epidermidis</i>	C960, ATCC14990	Standard strain (ATCC)
<i>Staphylococcus epidermidis</i>	C621	Clinical isolate (from David Speert – Children's)
<i>Bacillus subtilis</i>	C971, ATCC6633	Standard strain (ATCC)
<i>Aeromonas salmonicida</i>	99-1, A449	Field isolate being sequenced at IMB
<i>Aeromonas salmonicida</i>	<u>97-4</u>	Field isolate
<i>Candida albicans</i>	C627, CALB105	Yeast test strain

Table 5. Sizes of introns (in bp) in genomic sequences amplified using primers PL5' and PL3'

Gene	Exon 1 Total	Intron 1	Exon 2	Intron 2	Exon3	
WF1	154	539	31	95	82	901
WF1a ¹	103	?	31	?	82	?
WF2 ²	100	525	31	108	49	813
WF3	100	374	19	97	64	654
WF4 ²	100	230	31	101	49	
	511					

¹Intron sizes could not be determined as this sequence is only represented by an RT-PCR product

²Sequences were also amplified using primer PL1 and PL2

Table 6. RT-PCR products from skin and intestine corresponding to different pleurocidin genes

Skin Band	Intestine	Size	
4	n/d ¹	265bp	
WF1			
5	2	175bp	
WF2			
4	9	175bp	
WF3			
n/d ¹	n/d ¹	-	
WF4			
n/d ¹	7	215bp	n/d ²

¹not detected

²not detected by genomic PCR (corresponds to WF1a)

Table 7. Sizes of bands (in kb) hybridising to
pleurocidin probes in *Bam*HI and *Sst*I digests of winter
flounder DNA

Probe	<i>Bam</i> HI	<i>Sst</i> I
WF1	>24, 6	19, 17, 4.5, 4.4, 3.0, 2.9, 2.2, 1.3, x
WF2	6	19, 17, 4.5, 4.4, 2.9, x 1.3, x
WF3	>24	19, 17, 4.5, x 2.9, x 2.2, 1.3, x
WF4	17, 6	19, 17, 4.5, 4.4, 2.9, x 2.2, 1.3, 1.2

x=no hybridising band evident

Table 8. Minimal inhibitory concentrations of pleurocidin-like cationic antimicrobial peptides against a wide spectrum of bacterial pathogen and *Candida albicans*. Pathogens were grown in Mueller-Hinton broth and exposed to a range of concentrations of the

	<i>A.sal</i> 99-1	<i>A.sal</i> 97-4	<i>S.typh</i> MS4252 s	<i>S.typh</i> 14028s	<i>P.aeru</i> K799	<i>P.aeru</i> Z61	<i>E.coli</i> C786	<i>E.coli</i> UB1005	<i>E.coli</i> DC2	<i>S.epi</i> C621	MRSA C623	<i>C.alb</i> C627
NRC-1	64	64	16	>64	>64	32	32	32	32	>64	>64	64
NRC-2	>128	128	64	>64	64	32	64	64	64	>64	>64	>64
NRC-3	2	4	2	8	2	1	2	8	2	8	8	4
NRC-4	2	2	2	16	8	4	2	4	2	8	8	8
NRC-5	>64	>64	64	>64	>64	32	64	64	>64	32	32	>64
NRC-6	4	4	4	64	16	4	4	4	2	>64	32	32
NRC-7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NRC-8	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
NRC-9	>64	>64	64	>64	>64	64	64	>64	>64	>64	>64	>64
NRC-10	>64	32	16	>64	32	8	32	32	32	32	64	>64
NRC-11	8	8	4	32	32	4	4	16	4	64	>64	32
NRC-12	2	2	2	8	4	1	2	8	2	8	16	4
NRC-13	4	2	2	8	4	1	2	4	2	4	4	4
NRC-14	32	16	16	>64	32	8	16	16	16	16	16	>64
NRC-15	8	16	4	16	8	4	8	8	8	4	4	16
NRC-16	2	1	0.5	16	4	1	1	2	0.5	16	32	8
NRC-17	2	1	1	8	4	2	1	4	1	32	16	8
NRC-18	>64	128	32	>64	>64	64	64	64	64	>64	>64	>64
NRC-19	64	>64	16	64	32	8	32	16	32	8	8	64
NRC-20	>64	>64	>64	>64	>64	64	>64	>64	>64	>64	>64	>64

specified peptide. The lowest peptide concentration which inhibited bacterial growth by at least 50% was recorded as the minimal inhibitory concentration.

Table 9. Characteristics of winter flounder and Atlantic salmon hepcidin-like peptides

Name	Total Amino Acids	Total Cysteines	Molecular Weight	pI
WF1	27 8.75	8	3066	
WF2	19	6	1992	5.54
WF3	22	8	2367	8.74
WF4	22	8	2256	8.52
Hb5.3	22	8	2363	8.75
Sal8.6	22	8	2331	8.76
Hb17	22	8	2391	8.76
Hb1.1	22	8	2391	8.76
Hb357	22	5	2397	7.84
Hb7.5	25	8	2881	8.53
Sal2.1	25	7	2925	8.60
Sal1	25	8	2720	7.73
Sal2	25 8.53	8	2881	

Table 10. Semi-quantitative RT-PCR analysis of hepcidin expression in Atlantic salmon during bacterial challenge

Tissue	Type I Hepcidin			Type II Hepcidin		
	Control	Infected	Ratio	Control	Infected	Ratio
Esophagus	nd	0.08	↑	nd	0.09	↑
Stomach	nd	0.09	↑	nd	0.27	↑↑
Pyloric caecae	nd	0.14	↑	nd	0.37	↑↑
Liver	1.19	2.36	2	nd	1.45	↑↑↑
Spleen	nd	0.18	↑	nd	0.41	↑↑
Intestine	nd	0.21	↑	nd	0.33	↑↑
Brain	nd	nd	0	nd	0.50	↑↑
Blood	0.82	0.84	1	nd	nd	
Anterior kidney	0.06	0.07	1.2	nd	0.08	↑
Posterior kidney	0.07	0.14	2	nd	0.11	↑
Gill	0.13	0.12	1	0.08	0.07	1
Skin	0.14	0.18	1.3	0.07	0.09	1.3
Ovary	nd	nd	0	nd	nd	0
Rectum	0.07	0.13	2	nd	0.08	↑
Heart	nd	nd	0	nd	0.43	↑↑
Muscle	0.38	0.8	2.1	nd	0.60	↑↑

Pixel densities obtained by densitometry are expressed relative to the actin signal. The ratio of infected:control was calculated where numerical values were obtained for both conditions. nd, not detected; ↑ weakly up-regulated; ↑↑ strongly up-regulated.

Table 11 (Cont.)

Signal peptide	Anionic propeptide	Mature peptide	NRC Code
		---SFQKQKFCGCGCRA-GVQGLCQKF	NRC224 ^b
		---GFKCKFCGCGCRP-GVQGLCQRR	NRC225 ^a
		---GFKCKFCGCGCRP-GVQGLCQRF	NRC226 ^b
		---GFKCKFCGCGCSP-GVQGLCQRF	NRC227 ^b
		---GFKCKFCGCGCSP-GVQGLCQKF	NRC228 ^b
		---GFKCKFCGCGCRP-GVQGLCQKF	NRC229 ^b
		---GFKCKFCGCGCRP-GVQGLCQKF	NRC230 ^a
		---GFKCKFCGCGCRP-GVQGLCQRF	NRC231 ^a
		---SFQKQKFCGCGCRR-GVQGLCQKF	NRC232 ^b
		---GFKCKFCGCGRG-ALCGLCQKF	NRC233 ^b
		---GFKCKFCGCGRG-ALCGLCQKF	NRC234 ^b
		---GFKCKFCGCGRG-ALCGLCQKF	NRC235 ^b
		---SADQWPGGNQ---CGTCQKV	NRC236 ^a
		---SACSFCCNES---GGICQKF	NRC237 ^b
		---SACSFCCNES---GGICQKF	NRC238 ^b
		---GSNCKPCGNHN---GGTCCEV	NRC239 ^b

• peptide predicted from expressed tag and/or RT-PCR product

^a Peptide predicted from genomic sequence

- deletions introduced to clarify alignment conserved cysteines are shaded

conserved cysteines are shaded

Table 12. Nucleotide sequences of pleurocidin-like genes and cDNAs referred to in Table 11.

Winter Flounder WF1

ATGAAGTTCACGCGCCACCTTCCTCCTGTTGTTTCATCTTCGTCCTCATGGTTGATCTCGGAGAGGGTTCGTCGTAAGAAAAAGGGGTC
GAAGAGAAAGGGGTCCAAGGGAAAGGGGTCCAAGGGAAAGGGCAGGTGGTTGGAAAGGATTGGTAAAGGTAGAGTCACGGAATTAA
TTTGCCTTTTACATTGCAAAATATTTTTCATATAACATTGCTGGAAAATCACAAAATAAGTAGTCAATATATTTGGCCAAATAGAA
TCACCTTGATTTCATAATAATCAAAATAACAACCTAAAAGGCCCTTGATTAGCATGTTCCCTCAATGAAATGGACATTGTAATTT
ACCTTGATTCTCACATGCTACGACCTGCTGCAGCAACATTGAAAATAAATTTGTCCAGAAAGATTTTAAAGTACATTGTTATAGG
CGATTATCTTTCTATTAATCAGATATTTGTTCAAACCAATAGAATAACTGGATCTCTATGCTAAAATAATAAAACACACATTACG
ATGTTACCAAGTCAAGATTGAACGCTGTTTAAAAGTAAGTATGAAACATCCTCTGTATGTATAATTTGTTTAACTGGTAACTTATAGT
CCTAATAATTGCGTTATGAAATGTATTAATTGTCATTTAATATAATTTGCTGGAATTTATCAGTGTGTTTGTGTTTGTGTTTAA
CACAGCTGGCGGATAATTATCGGGGGGGCCCTTGAGTAAGGACTTCTACCATCATTACTGTGTAATATTTATAGTTATGATCAGT
ACAGTTATTAACAACCTTCCTTGTCTCGCTGAACCTTCTCCATCAGTCACCTCGGGCAGGGGCAGGTGCAGGGGCGGATTACGACT
ACCAGGAGGGGGAGGAGCTCAACAAGCGCGCAGTCGATGAA

//

Winter Flounder WF1A

ATGAAGTTCACGCGCCACCTTCCTCCTGTTGTTTCATCTTCGTCCTCATGGTTGATCTCGGAGAGGGTTCGTCGTAAGAGAAAGTGGTT
GAGAAGGATTGGTAAAGGTGTCAAGATAATTGGCGGGGCGGCCCTTGATCACCCTCGGGCAGGGGCAGGTGCAGGGGCAGGATTACG
ACTACAGGAGGGGCAGGAGCTCAACAAGCGCGCAGTCGATGAA

//

Winter Flounder WF2

GCCCACCTTGTATTGCGAAGGTAATATTGATATTTTTCATATTCATTTAGACAAAATGTGCTCAGCTTGTTACTGTATAATGCAAAA
GTTAATGATCTTTATTTTTCGTTTTCCTGTTAGAGATGAAGTTCACGCGCCACCTTCCTCATGATGCCATCTTCGTCCTCATGGT
TGAACCTGGAGAGTGTGGCTGGGGAAGCTTTTAAAAGGGCTGCTCAGGTAGAGTCACAGAATTAATTAGCTTTTGGCTTTGCA
AATATTTTATAACAGCTGGAAAATCACAAAATAAATAGTATATATTTGGCCAATAAATCACTTTGATTTCATAATAA
TCTAATAACCAACCTAAAAGGCCCTTGATTAGCATGTTCCCTCAATGAAATGTACGTTGAGGTTTATTTTGATTCTCAAGCAC
CAACCTGCTGCGTCAACAATTGAATTCAAATTTGTCCCAAAGGAATTCAAAGTAAATTTTCTAGGCGATTAACTCTTCATTAC
TCTGATTGTTTAAAATAATAGAAATACTCAATCTCTATGATAAAAATACACATACATTGAGATTTTATAGGACAAGATTG
AAAACCTCTTACAAGTATGTATAAAACATCATCTGTATGTATAATTTGTTTAACTAGCTTACCTACTAATTTGTTTAAAT
TGTCAATTAATCAATTGCTTGAGTTTATCATTTATGTTTGTGTTTTCACAGTTGGCAAGCATGTTGGCAAGGCGGCC
TTACGTAAGGACTTCTACCATTTTACTGTATAATTTTGATAGTGTATCACCAGTACTGTTTTCACAACTTCTCTATTCTCTGCTG
ACTCTCTCCATCCGACTCATCCGAGTCATTACCTTGGCGATAAGCAGGAGCTCAACAAGCGTGCAGTCGATGAAGACCCAAATGT
TATTGTTTTGAATGAAGAAAT

//

Winter Flounder WF3

ATGAAGTTCACGCGCCACCTTCCTGGTGTGCTGCTCCCTGCTGCTTAATGGCTGAGCCTGGAGAGTGTTCCTTAGGAGCCCTTATCAA
AGGGGCCATACATGGTAGAGTCAAGGAATTAATTAGATTTTTCATGTCAATTAATGTAGTAGAACATATATAAGTAGTCAATATA
TTTGACCAAGTAGAATCATTGTTTCAATAATAATCAAAATAACAATCTCCAGGCGATTAAATATTGCAATAATTGATTGTTA
TAGAATACGGAACAACCTGGATCTTAATGCTAAAATAATCAACATACATTCTGATTGTCAGGCAAAATTAACACTACTTTAA
GTATGTATAAAACATAATCTGTATGTATAACAAATACTCCAAGCAATTGTGTATGAAATGTATTCAATTGTCAATTAAATATAAT
TTGCTTGAGTTTATCATCTGTGTTTGTGTTTGTGTTTTCACAGGTGGCAGGTTTATCCATGGGTAAGGACTTCTACCATCATGAC
TGTTATTTTAAATATTATTATCATCAGTACTGTTATTGACAACCTCACTTGTCTCGCTGACTCTCTCCATCAGAATGATCCAAA
CCATCAGGTTATGACGAGCAGCAGGAGCTCAACAAGCGCGCAGTCGATGAA

//

Winter Flounder WF4

GCCCACCTTGTATTGCGAAGGTAATATCAATATTTTCAAATTCATTTAGACGAGACCAACCTTTTGGGAAATCTGCTCAGCTTAT
TACTGTATAATGCAATGTTAATGATCTTTATTTTTCGTTTTCCTGTTAGAAATGAAGTTCACGCGCCACCTTCCTCATGATGTT
CATCTTCGTCCTCATGGTTGAACCTGGAGAGTGTGGTTGGGGAAGCAATTTTAAGCATGGTTCGTCATGGTAAAGTCACGGAATTAA
TTAGCTTTTAACTTTGCAAAATATTGTTTTCCTTAAACAGCTGGAACTCAAAAATAAATAGCCGATATATTGGCCAATTAT
AATCACTTTGATCTAAATAACAACCTAAAAGGCCCTTGATTAGCATGTTTCTTCAATAAAATGATTGAACACTACTTAAAGGTATG
TATAAAACATCATCATGTGTTTGTGTTTGTGTTTTCACACAGCTGCCAAGCATATTGGCCATGCAGCCGTTAAGTAAGGACTTCTACC
ATTATTACTGTATAATTTGATAGTATATCACCAGTATGTTATTGACAACCTTCTCTTTTCTGCTGATCCGACTCATCCGAG
TCATTACCTTGGCGAGCAGCAAGATCTCGACAAGCGCGCAGTCGATGAAGACCCAAATGTTATTGTTTTGAATGAAGAAAT

//

Yellowtail Flounder YT2

ATGAAGTTCACGCGCCACCTTCCTCATGATGTGCATCTTCGTCCTCATGGTTGAACCTGGAGAGTGTGCTTGGGGGAAATGGTTTAA
AAAGGCCACACACGGTAGAGTCACAGAATTAATTAGCTTTTGTCTTGGCAAAATATTTTATAACAGCTGGAAAATCACAAAAT
AAATAGTCTATATATTGGCCAATTAGAATCACTTTGCTTTCAATAAAAATCTAAATAACAACCTAAAAGTCTTTGATTAGCATT
TTCCATCAATGAAATGGAGCTTGAGGTTTATTTTGAATCCACATGACCGGCTGCTATGTCAACAATTGAATACAAATTTGTCC
CAGAGGAATTCAAAGGAAATTTTCTAGGCGATCTAATCTTTCCATTACTCGGATTGTTTAAATATATAGAAATAACTCAATCT
CTATGATAAAATAATAACACATACGTAAAGATTTTACAAGACAAGATTGAAAACCTTCTTAAAGTACGTATAAACATCATCTGT
ATTTATAATTGTTTAAACATTAAACAATAGCCCTACTAATTGTGTTATGGAATGTATAAATGTTCATTTAACATAACTTGTGTTGA

GTTTATCATTATTTGTTTTGTTTGTGTTTTACACAGTTGGCAAGCATGTTGGCAAGGCGGCCCTTACGTAAGGACTTCTACCATCA
TTACTGTATAATTTGATAGTATTATCACCAGTACTGTTATTGACAACCTCTCTTGTCTGCTGACTCTCTCCATCCGACTCATCC
ATAGTGCTTACCTTGGCGACAAGCAAGAACTCGACAAGCGCGCAGTCGATGA

//

Winter Flounder WFX

TAATAAACTAATGTGTAAAGTCTTCCACTTTTTTACTGTATTTACTTAAACAGAAAATTATCTCACGATTCTGGAGCTGCAGC
CACTAAGTGTGTGCTTCATGAAGTGAATACCAATTGTTCTAACAACCACTCAACCAATTAACCAGAATCTACAAAGTGAGGAAGTG
AGAGGAGTCGTCTGTGTTTTCAAAATTTTTGAATGATCTACCCTATGTGAGCTCCTCCTGTTATAGCTCTAAATGTTACACAAAT
GAATGTGAAGTCAGTTCTGTGTATATAAAGAGTTGCCCTGTAGAGCATAACAAGATTTACCTTTGAATCTCACAAACCTCACT
TTGTATTTCGACAGGTAAGATCGATATTTTCAAACTCATTIAGACGAGACCAAGTATTTGGGAAATGTGCTCAGCTTGTCAATGTA
TAATGCAAATGTTAACAATCGTTTTGTCTTATGTTGTGTTTGTAGGATGAAGTTTCGCTACTGCCCTCCTGATGTTGTCCATGGTC
GTCTCATGGCTGAACCTGGAGAGTGTCTCTACAGAGGACATCATCAAGTCATCTCGGGTAGAGTCCAGGAATTAATTATTAT
CAATAACAATGAAATAACAACCAAAAGGCTCTGATTAGCATGTTCTTCAATGAAATGGTCTGTTTTTATCTATTTTGATTCTCA
CATGCAACGACCTGCTGCGGCAACATTTGAAAATCAATCTTTTACACAAATTCAAAGTACATTGATTTATTTCGATTTAATCTTA
ACATTAATCAGATTGTTTTGTTTTAAATATATCGAATACTGGATCTCTATGATAAATAATTAACATACATTCTTATTTTACC
AATCAAGATTGAACACTTCTTAAAGTACGTATAAAACATCATCTGTATGTATAATTGTTTGATTGTTAAGTAATATTTCGAATAA
TTGTGTAATGGAATGTATTAAATGTCATTAAATATAATTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTAAACAGGT
GGAGGTTTTCTCAATGCGTAAGGACTTCTATCATCATTACTGTGTAATTTTTATAGTATTATCATCAGTACTGTTATTAACAGCTT
CTCTTGTCTCACTGACTCTCTCCATCAGAAATGAACGCGGTTACAATGAGCAGCAGGAGCTCAACAGCGCTCAGATGATGATGAC
AGCCCCAGTCTTATTGTTTTGACTGAAGAAGTCGCCCTGAAGGAGCCTTCAGATGATATATTATGCTTCTTGCTCTTCATTGAAA
TAAATCAAAC

//

Winter Flounder WFY and WFZ (alternative splice products from the same pseudogene)

GAGCTCGATCAAACCAGACAAAGTTGCCTTCCCTTCAACAATAGAGTGAAGAGAAAACAGGAGAGGACTTGTATCTCTCTGATG
CTGAGAAGAAGAAATAAGAAAGACTTGCAGCATTTGATACTTTTACTTATACAGAAAACCTATAAACATGACGGGAGCATAAGTTAA
AGTCACAATACAGAAGAGAACCCAGAACCTGCAGCAAAATTTACTGGTATTCATATGATACTGGAGCCAAAGCAACGCAGAGA
CTCAGCAGCAGTGAACCAAGAGTTAACTGTACTTGTGTCCAGGTTGAATGAAAGTATTGAATAAAAAAACCAAGACAGAACAT
GCATATTTTTTGGAAATGGAATATAAGTCAGGAGAATATGTGTTGTTGTTGGTGGCAGGATCCATCACTCTGTCAAGTTAACACAAG
AACTTTTAGAAAACATAGATACGATCTCAAGTAACTTCCATTACTATTGACTTTTTTAAATACTTACAAATTTATTTTAA
AGCAACAATAAATCAGAGATAAATCTCATGGAGAAGTCTATATTCATATTTGTGAGCTGAACATTTCATGCTGCTGTTCTATCAT
CTGAGTGTGGAGGCCACTGACGTTTACTGACCTCAACGCTCTACCGCTCTAATGCATTGGAGTTAAAGGTAAGCAATTTGTTATTT
GTCTTCACTGTATTGATACTAAATATACAGGGTTACAAATACAGTTAAACAAGAGAGACGAGGTTGCGAAAGCTTCAGCATCAAT
GTGCTGATCGCTGATAGCTGATCTTACCCGACACCGGTGACATGGCATCAAAATGACCACCTCTTTTTCTCTCTTTTTTTGTA
GGACGAAGTTCCGCTGCCGCTTCCCTCGTGTGTTTCATGGTCATCGTCATGTTTGAACCTGGAGAGTGTTTTTTAGATTGCTTTTT
CACGGGGTCCACCATGGTAGGGTCCCGAAGTAAATTTGATTATTTACATGCCAAATATTTAATGAAACATACCTTATGAGTAGTTG
TATTTATTTGGACAAGTAGAATCTCTATGATTTTCACTAGTAATTTAGAATAACAATCAAAAGGCCCTTTGATTAGCATGTTCTTCAA
TGAAATGGACATTGAGGTTTATTTTGTATTCTCACATGCTACAGCAACAATTTGAATCAAAATTTTTCGCAAGAAACTTAATTAAC
ATTGTTGTGCAATAGTGCTTAAAGAGTGTACCATGGAATGGTGTGCGTTTAGGCACCTCAATAAATTTGGTTATCAAAATTAAT
AAAAAATTAATATTTAAATATTAATATTAATCATAACTTTAATTTGTTTAAAGTTTCTCGCGGGAAACCCCTTCTCTGAAGG
TAAAGGATAGCCAATTTATTGATTAAAGATCAGTCTCATTAGATCTAGTTTCAAATAGAAATCTCAATATTTTACCATCGAAGATTT
TATAATGAACAGTGAAGTTTATGGAGTTCTAAACAGTGTAAACAGTTGGCAAAGTTCACTATTGCAATATTAATGACAGACCATTTG
TGAAGAAGAACATTTATTATGAGCATAATAAAGTATGAAAGCACGAATTAATAACAATCAAAGCTAACTAAACAAGGACGTGTGT
GGGTGTGTGTGTAATGTAAATAAGCGGGGGGCTCAAACCTGGTGGCTACAAGAAGAGCCTTAAGATAGCAACCACAAGGCTGTA
CCATAAATGTTGTAGTAAAGAGTTATTAATAGATGTAGAATAACTAGTACTAATAGTACAACTAGTACAACTAATAA
CACTAAACAATAACAAGGAAGTGTGTGTGAGTGTGTTGTGTGTTAAATGTTAATTAGGGGCTCTCAAACCTGGTGTCTTACCAGAAG
AGTAAGATAACAATTTCCCCCTTCTCTGAGGTTGTTTTACGACTGTGTCTTTATGGCCGTGAGGGAAGGTTAACTCGGTGACA
TGCTATACGTGTCTGTGTAGATGTTAATCAGAGAATGCCAGAGTCAGAGAGACCTACGGAGGAAGTCTGTGAAGGGCCTATCTAAC
ATTAGCTTTCTTTTAACTTATAACACAATATCAGAAACACATATCAACCTTATAAACACACAGAAATCAATAAACAGTCTTGCT
TAGCATGTATAATTATTAAGCCAGATTATGTTACCACTCGAGGGAAGAGTTTCAAGTGTGCTGAGTGCAGGTTGAAGTTCTCTGAGGACATCGCGTCTG
TGGTCTGATAGTTCTGCTATTCGCGATTCTGTGAGCCGTGTGCTCAGATGCAGGTTGAAGTTCTCTGAGGACATCGCGTCTGCTG
CGAGGATTTTGTAGAGCTTGAAGGGCGAGGAGATTTCCCTGAGTGGTGAAGCTGGAAGCTGGACCTCTGACCTCTGCTGTTGTTGTTG
GAAGAGAAGAAGCTGGAGCGGCGTGTCTCTCCCTTAGCCGATGAGGAGGAGAAGCCGCGCAGCCCCACTCTTGAAGAGTTGT
GGAGAGAGATGGGAGCAAGAGCTAGATTTTGGGGAGACCTCTCTTATTTGGCCCCGATGACCTCACAGGCTTGGAAACGGAGT
GACCAATAGGAGTTGACCTTGGTAATCTTGACACCTTTGTGGGACATTTGAGACCTTGAAGACCCAGGACATGAGCTGTTTCAATC
TGGGAGACGGAGTTCTTGACTGTCTCAGAAACATGAGAACCTGTGGCATCTTGGGGATTTAGTCCACTCGAGCAGATGCGGCAT
GTTTGTTCAGAGTTTGAAGTGAAGGAGGCTGTGGTTTGCACAAAACCATGTCCCAACAACATTTCTAGGCGATTTAATCTTTA
CATAAATTTGATTTGTTTTTAAATAATATAGAAATACTCGATCTTTCTGCGTAAATAATAAAAAATAAATTTCAATTTGACCAAT
CAAGATTGAACACTAATGAAGAGTACCTATAAAACATAATCTGTATGTATAGTTGTTTGAAGTGTAAATAGTAGTCTTAAACAAATG
AATGCGTAGGACTTCTACCATCATCTAGTGTAAATTTAGTTTATCATCAGTACTGTTATTGACAACGCTCTCTGTCTTGC
TGACTTGACTCTCTTCATCAGATTAAACCCAGGGCCGTTACAATGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGACAACCT
CAGTGTCTATTGTTTTTACTGAAGAAGTCGACCTGAAGAATCTTTTGAAGTATATGAAATGTTTGCCTTTCAATGAAATAAATCA
AACATGACTGGATATTTGTTCTTTTCATTGATGATTGTTGAGTGACAGTTGAATAATTTTGGAAACCTTATAACAGATCTCAAT

//

GCCCACTTTGTATTTCGCAAGGTAAGATCAATATTTTTCAAATTCATTAGACGAGACCAACCGTTTTCGAAATGTGCTCAGCTTGT
 TATTGTATAATAACAAAGTTAACGATCTTTATTTTTCTGTTTTTTTGTAGAATGAAGTTCAC TGCCACCTTCCTGATGTTGTTTCA
 CTTCGTCCTCATGGTTGAACCTGGAGAGTGTTGGATTGGAAAAGTGTGTTTTCGTAAGGCTAAGAAAGGTAGAGTCACGGAATTAATTA
 GCTTTTTACATTGCAAATAGATTTTTTATAACAGCTGGAAAAATCACAAAATAAATAGTCGATATATTTGGCCAATTAGAATCACT
 TTAATTTCAATAATAACTTAATAAACACTTAAAGGCCCTTTGATTAGCATGTTTCTTCAATGAAATGGACATTGAGGTTTTATTTT
 TACTTCTCAGATGCACCGCACTGTGCGGCAACCATTTGAATTCAGATTTGTCCAGAGAATTCAAAGTACATTTTTCCAGGCGATTA
 AATCTTTCCATTACTCAGATTCAAAAATAAATAAATGGAATAATTGAAGCACTATGATAAAATAATTACACATTCACTCTGACTTT
 ACAAGTCAAGATTGAACACTATTAAAAAGTGTGTATAAAACAACATCTGTATGCATAATTGTTTTAACTGTTAATAGTCCTAATTAAT
 TGTTTTATGGAATGTATTAAATTTACATTTAATATTATTGTCTTGAGTTTACCATCATGTTGTTTTGTTTTGTTTTACACAGTTGG
 CAAGACTGTTGGCGGCTTGGCCCTTGAGTAAAGGACTTCTACCATTACTGTATAATTTTGATAGTATTATCCACAGACTGTGTA
 TTAACTACTTCTCTTGCTGTGACTCTCTCCATCCGACTCATCTGCACTTACCTTTGGCGAGCAGCAGGAGCTTGACAGCGCG
 CAGTCGATGAGGACCCCGATGCTATTGTCTTTGACTGAAGAAGTCGCTTGAAGGAG

//

ACTTTGTATTTCGCAAGGTAAGATCAATATTTTTCAAATTCATTTAGACGAGACCAACCGTTGGCGAAATGTGCTCAACTTGTATT
GTATAATAACAAGTTAACGATCTTTATTTTTCTGTTTTTTGTAGAATGAAGTTCAC TGCCACCTTCCTGATGTTGTTTCATCTTC
GTCTCATGGTTGAACCTGGAGAGTGTGGATGGAAAAAATGGTTTAATAGGGCTAAGAAAAGGTAGAGTCA CGGAATTAATTAGCTT
TTTACATTGCAAAATAGATTTTTTATAACAGCTGGAAAAATCAAAAATCAAAATAGTCGATATATTTGGCCAATTAGAATCACTTTAA
TTTCAATAATCTAAATAACAACCTAAAAGGCTTTGATTAGCATGTTTCTTCAATGAATGGACATTAGAGTTTATTTTGATCTC
ACATGACCGCAGCTGTGCGGCACCACTTGAATTCAGATTTGTCCAGAGAATTCAAAGTACATTTTTCCAGGCGATTAAATCTTT
CCATTA CT CAGATTCAAAAAATAAATAAATAGAATAATTGAAGCACTATGATAAAATAATTACACATTCAC TCTGATTTTACAAGTC
AAGATTGAACACTATTAAAACTGTGTATAGAACATCATCTGTATGTGTAATTGTTTAACTGTTAATAGTCCTAATAATTGTTTTTA
TGGAATATGATTAATTTACATTTAATATTATTGTCTTGAGTTTACCATCATGTGGTTTTGTTTGTTTTTACACAGTTGGCAAGACT
GTTGGCGGCTTTGGCCGTTGAGTAAGGACTTCAACATCATTA CTGTATAATTTTGATAGTATTACACGAGTCTGTTATTAACTA
CTTCTCTGTCTCGCTGACTCTCTCCATCCGACTCCTCTGAGTCACTTACCTTTGGCAAGCAGCCGGAGCTCGACAAGCGCGCAGTC
GATGAGGACCCCACTGCTATTGTCTTTGACTGAAGAAGTCGCTTGAAGGAGCCTTCAGAA

11

TTGCCCACTTTGTATTTCGCAAGGTAAGATCAATATTTTTCAAATTCATTAGACGAGACCAACCAATTTGGGAAATGTGCTCAGCTT
GTTACTGTATAATGCAAAGTTAAGTATCTTATTTTTTCGTTTTTTTTTTGTAGAATGAAGTTCACATGCCAATCTTCTCATGTTGT
TCATCTTCGTCTCATGTTTGAACCTGGAGAGTGTGGTTGGCGAACATTGCTTAAAAAGCTGGTCAACGGAAATTAATACGCTTTTT
ACATTGCAAATAGATTTTTTATAACAGCTGGAAAAATGACAAAAATAAATAGTTCGATATATTGGCCAATTAGAATATATTTTGAATTT
CAATAATAATCTAAATAACAACTAAAGGTCCTTGAATTAGCATGTTCTTCAATGAATAAGGACATTGAGGTTTTATTTTGATTCTC
ACATGACCGACCTGCTGCGCGCAAAATGAATTTCAGATTTGTCCAGAAAGAAATTCAAAGTAAATTTTCCAGGGGATTAAATCTTTC
CATTACTCGGATTTAAAAAATAAGTAATAACTGAATTGCCATGAAAAATAATTACACATACTGTCGTGATTTTACAAGTC
AAGATTGAACACTACTTAAAAGTATGTATAAAACATCATCTGTATGTATTAATGTTTAACTGTTAAACAAATAGTCCAAATAATTGT
GTTATGAAATGTATTAAATGTTCATTAAATATAATTTGCTTGAGTTTATCATCATGTGTTTTTTTTTTTTTTTACACAGAGGTTA
AGACTGTTGGCAAGTTGGCCCTTAAGTAAGGACTCTACCAATCATTACTGATAGAAATTTGATAGTATTATCACAGTACTAGTAT
CTGACAACTTCTCTTCCCAACCATCATCCGAGACATTACCTTGGCAGTACGCGGAGCTCGACAAGCGCGCAATTGATGACGA
CTCCAGTATATTGTTTTTGACTGAAGAAGTCGCCTTGAAGGAGCCTTCAGAA

14

ATGAAGTTCACCTGCCACCTTCCTCATGATGTTTCATGGTCGTCCTCATGGCTGAACCCGGAGAGGCTGGTTGGGGGAAGTATTTTCRA
ACATATTTTCAAAGCTGGAAAGTTTCATCCATGGTTCGATCCAGGCACACAATGACGGCGAGGAGCAGGATCTCGACAAGCGCGCAG
TCGATGA

/

ATGAAGTTCACTGCCACCTTCCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGGGTTTTTGGGGAAAGCTTTTGAA
ATTGGGCATGCATGGAATCGGGCTGCTCCATCAGCATTGTTGGTGCTGACGAGCAGCAGGAGCTCGACGAGCGCTCAGAGGAGGACG
AGCCCAATGTTATTGTTTTTGAATGAAGAAGTCGCATTGAAGGAGCCTTCAG

/

ATGAAGTTTCACTGCCACCCTTCCTGGTGTGTTCATGGTCGTCTCATGGCTGGATCCGGAGAGTGTGGTTTGAAAAAAGTGGCTCCG
TAAAGGTAGAGTCATGGATTTTAATTTGCTTTTTCATTGCGAAATACCTTTAATAATACATAGTTGGAAAACACAAAAATAAGTAGT
CGATATATTTGGCCATATAGAATCACTTTGATTTCAATAATAATACAAACAACATCAAAAGCCGATTTGATTAGCATGTCCCTTC
ACTAAAATTGGACATTTGTAATTTATTTTGATTTCTACAGGCACTAACCTGCTGCGGCAACAATTGAAATCAAATTTGTCTCAGAAGA
ATTCAAAGTACATTGTTCTAGGCGATTTAATCTTTCCATTCACTGGATCTGTTTTTAAAAATATAGAATAACATCGATCTCTATGTT
AAAATAATAAAACACACATCTCGATTTTACCTGTCAAGATTGAACAACGACTAAAAGTAGTATAAAAACATCATCTGTATGTATAA
TTGTTTAACTTGTCACACTAATAGTCCAAATAATTGTGTTATGGAATGTATTCACTGTGCATATAATATCATTTGCTTGAATTTATCA
CCATGTGTTTTTGTGTTTTTGTGTTTTTACACAGGTGCCAAGCACTTTGGCCAGGCGGCCATTAAAGTAAGGACTTCTACCATCATTACTGTG

TAATTTTAAAGTATTATCATCAGTACTGTTATTGACAACACTACTCTTGTCTCTGTTACTCTCTCCAGGGGTTTGGCCTCTTGCGAA
GAGCAGCAGGAGCTCGACAAGCGCTCAATGGATGACGAGCCAGTGCTATTGTTTTGACTGAAGAAGTCGCCTTGAAGGAGCCTT
CA

//

Witch Flounder GC3.2

ATGAAGTTCACITGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGTTCAC
TAAAGGTAGAGTTCATGGATTTAATTTGCTTTTTACATTGCAAAATACCTTAAATATAACATAGCTGGAAAAATCACAAAAATAAGTAGT
CGATATATTTGGCCATATAGAATCACITTTGATTTCAATAATAATCAAAACAATAATCAAAAAGCCTATTGATTAGCATGTTCTCTTC
ACTAAATGGACATTGTAATTTATTTTGATTCTCACAGGCACCAACCTGCTGTGGCAACAATTGAAATCAAATTTGTCTCAGAAGA
ATTCAAAGTACATTGTTCTAGGCGATTTAATCTTTCCATTCACTGGATTTGTTTTCAAAAATATAGAATAACTGGATCTCTATGTT
AAAATAATAAAACACATTTCTGATTTTATCTGTCAAGATTGAACACGACTTAAAGTATGAATAAAACATCATCTGTATGTATAATT
TTTTAAGTGTCAACTAATAGTCCAAATAATTTGTGTTATGGAAATGTATTCAATGTCTATATAATATCATTGCTTGAATTTATCACC
ATGTTCTCTTTGTTGTTTTTACACAGGTGAAAGGTTATCCAGAGGTAAGGACTTCTACCATCATTAATGATAATTTAATAGTA
TTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTCGCTGACTCTCTCCATCAGGCATTTGCTGACGTGAGCAGCAGGAGCT
CGACAAGCGCTCAGTGGATGACGAGCCAGTTCTATTGCTTTTGACTGAAGAAGTCGCCTTGAAGGAGCCTTCAG

//

Halibut HB26

TTATGAAGTTCACITGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAGCCTGGAGAGTGTTTTTTGGGATTGCTTTTT
CACGGGTGCCACCATGTTAGGGTACCGGAAGTAATTCGATTTTTACATGGCAAATATTTAAGATAACACACCATATGAGTAGTCCG
ATATATTTGACCAATTAGAATCACITTTAATTTCAATAATAATCAATAACAATCTCTAGGCCATTTAATCTTTCCATTAATCGGA
TTTGTTTTTTTAAATATAGAATAACTGGATCTCTATGTTAAATAATAAAACATACATTCTGATTTTACCAGTCAAGATTGTACGC
TACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTAACTGTTAACTAATAGTCCAAATAATTTGTGTAATGGAAATG
TATTAATTTGTCATTTAATATCATTGCTTGAATTTATCACCATGTTGTTTTGTTGTTTTTACACAGTTGGAAAGTGGATCCATGG
GTAAGGACTTCTACCATCATTAATCTGTGTTATTTTAAATAGTATATCATCAGTACTGTTATGATATTTTCTCTGTCTCGCTGACT
CTCTCCATCAGACTCATCCATGGGCATCACGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCTGATGAAA

//

Halibut HB18

TTATGAAGTTCACITGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGGGTTTTTTGGGAATTTCTTTTT
CACGGGTGCCACCATGTTAGAGTACCGGAATTAATTCGATTTTTACATGGCAAATATTTAAGATAACACACCATATGAGTAGTCCG
ATATATTTGACCAATTAGAATCACITTTAATTTCAATAATAATCAATAACAATCTCTAGGCCATTTAATCTTTCCATTAATCGGA
TTTGTTTTTTTAAATATAGAATAACTGGATCTCTATGTTAAATAATAAAACATACATTCTGATTTTACCAGTCAAGATTGAACAC
TACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTAACTGTTAACTAATAGTCCAAATAATTTGTGTTATGGAAATGT
ATTAATTTGTCATTTAATATCATTGCTTGAATTTATCACCATGAGTTTTTTGTTGTTTTTACACAGGTGAAAGAGGCCCTTGCA
GTAAGGACTTCTACCATCATTAATCTTTGTTAATTTTATAGTATATCATCAGTACTGTTATGACAACTTCTCTGTCTCGCTGACT
CTCTCCATCAGGATGAACCTCAGAGCGTCGCAGTTACGACGAGCGGCAGCAGCAGCAGCAGGAGCTCGACAAGCGCGCAGTCTGATGA
AA

//

Yellowtail Flounder YT1

GCCACITTTGATTTCGCAAGGTAAGATCGATATTTTTCAAACCTCATTTAGACGAGACCAAGCATTGTTGAAATGTGATAAGCTTC
TAACTTTATAATGCAATGTTAAACAATCTTTTTGTTCTGTTGTTTTGTAGGATGAAGTTGGCTGCCGCCCTTCTGGTGTCTGTTCC
TGGTCTGCTTCATGGCTGAACCTGGAGAGGGTTTTCTGGGATTTCTTTTTACGGTATCCACCATGGTAAAGTCACTCATTTAATA
CATTTTTACATGGCAAATATTTGAATATAACATACTATATGAGTTGTCAATATATGTGGCCAAGTAGAAGCATTGATTTCAATA
ATAATCAAAATAACAATCACTAAGCCATTTAATAATTGAATTAATTAATTAATTTGTTTTAAAAAATATAGAATAACTGGATCTTTAT
GCTAAATAATTAACCTTAATTCAGATTTTACCACCTCAAGATTGAACACTACTTAAAGTATGTAAAAAACAATCATCTGTATG
TATAATTAATACTAGTCCAGTTAATTTGTTTTATGGAAATGTGTTAATTGACATATATCATTGCTTGAACCTTATAATGTGCTTTG
TTGTTTTTACACAGGTATCAGGGCGATCCATCAGTAAGGACTTCTACCATCATGACTGTGTTATTTAATAGTATATCATCAGT
ACTTTTATTAACAATCTCTTGTCTCGCTGACTCTCTCCATCAGTCTCATCCATGGTCAAAGATACGACGAGCAGCAGGAGCTTG
ACAAGCGCTCAGTCTGATGACAACCCCGGTGCTATTGTTTTTGACTGAAGAGCTGCGCTTGAAGGAGCCTTCAG

//

Yellowtail Flounder YT3

ATGAAGTTCACITGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGGGTTTTCTTTGGAGCCCTTATCAA
AGGGGCCATCCATGGTGGCAAGTTGCTCCATAAACTCATCAAAAAAACAATGAACATCACGGTTATGGCAAGCATTGGGGGCTTG
ACAAGCGCGCAGTCTGATGA

//

Winter Flounder WF-YT

TGAAAGTGAAGGAGTGAAGGAGGAGTACTAGTCTCTGTGTTTTCACTGCTTGAATTATCTAACACTATCTGAGCCCTTCTGCAATA
ACTCTAAATGTTACACAGTGAAGTCAAGTCTCTGTGTATATAAGAGTTGCATCTGTTGTTATCAGTAGACAACAGATTACA
CCTTTGAATCTCACAAGCTCATTTGTATTGACAGGTGAAGTCAATGTTTCAAACTCATTAGATGAGACCAAGCATTGTTGG
AAATGTCTCAGCTTCTAATCTGTATGATGCAATGTTAAACAATCTTTTTGTTCTGTTGTTTTGTAGGATGAAGTTGGCTGCCGCCCT
TCTGGTGTCTTCTGGTCTGCTCATGGCTGAACCTGGAGAGAGTTTTTGGGATTTCTTTTTCATGGTATCCGCCATGGTAGG
GTCACTGAATTGATACATTTTTACATGGCAAATATTTGAATGTAACATACTATATGAGTTGTCAATATATGTGGCCAAGTAGAAGC
ACTTTGATTTCACTAATAATCAAAATAACAATCACTAGGCCATTTAATAATGCAATTAATTACACTTGTTTTTATATAGAATATAG
AATAACTGGATCTTTATGCTAAAATTAATAAACATGAATTCAGATTTTAAGATTTTCAAGATTGAAAACCTACTTAAAGTATGTA

AAAAACATCATCTGTATGTATAATTAAATACCTTGTCCAGATAATTGTGTTGTGGAAATGTGTTAATTGACATATATCATTTGCTT
GAATTTATCATTATCTGCTTTGTTTGTGTTTTACACAGGTATCAAGGCGATCCATGGGTAAGGACTTCTACCTTCATGACTGTGTAT
TTTTAATAGTATTATATTCAGTACTGTTATGAAAACCTTCTCTGTCTCGCTGACTCTCTCCATCAGAATGATCCATGGTAACAGT
TTAGACGAGATGCAGGAGCTCGACAAGCGCTCATTCGATGACAACCCCAACGCAATTGTTTGTGACTGAAGAAGTCGCCCTGAAGG
AGCCTTCAGATGATATATAATGCTTCTTGCCTTTCAATGAAATAAATTGAATAATTACCCGCAACAGC

//

Winter Flounder WF1-like

TACTTTTATCTACCCTATGTGAGCTCCTCCTGTTAATCTTAAATGTTACACAATGAAGATGAGGTCAATTCCTGTGTATATAAA
GAGTTGCCCTCTGTATAGTAGACAACATATTTCACTTTGAATCCCAAAAGCTCACTTTGTACTCAACAGGTAAGATCGATATTTA
AAAACTAATTTAGACGAAACCAAGCATTTTGGGGAATTTGCTCAACTTCTAAATGTATGATACAAATGTTAACAATCTTTTATTTT
TGTGTTGTTTTTTGTAGGATGAAGTTCACCTGCCACCCTCCTCCTGTTGTTTCATCTTCGTCTCATGGTTGATCTCGGAGAGGGTC
GTCGTAAAGAAAAGGGGTGGAAGAGAAAGGGGTCCAAGGGAAGGGGTCCAAGGGAAGGGGAGGTGGTGGACAGGATTGGTAAA
GGTAGAGTCACGGAATTAATTTGCTTTTACATTTGCAATATTTTTCATATAACATTGCTGGAAATCAAAAATAAGTAGTCAA
TATATTTGGCCAAATAGAATCACTTTGATTTCATAATAATCAAAAATAACAACCTAAAAGGCCCTTTGATTAGCATGTTCTTCAAT
GAAATGGACATTGTAAATTTACTTTGATTCTCACATGCTACGACCTGCTGCAGCAACATTGAAAATAAATTTGTCCAGAAGATTT
TAAAGTACATTGTTATAGCGGATTTATCTTCTATTACTCAGATATTTGTTCAAACCAATAGAATAACTGGATCTCTATGCTAAAA
TAATAAAACACACATTGATGTTACCACTCAAGATTGAACGCTGTTTAAAAGTAAGTATGAAACATCTCTGTATGTATAATTGT
TTAAGTGGTAACTTATAGTCTTAATAATGCGTTATGGAATGTATTAATTGTCTATTAAATATAATTGCTGGAATTTATCACTGT
GTGTTTTGTTTTGTTTTTACACAGCTGGCGGGATAATTATCGGGGGGGCCCTTGAGTAAGGACTTCTACCATCATTAAGTGTAT
ATTTATAGTTATGATCAGTACAGTTATTAACAATCTCTTGTCTCGCTGAACCTTCTCCATCAGTCACCTCGGGCAGGGGAGGTG
CAGGGGCGGATTAAGCTTACGCTTACGAGGGGGGAGGAGCTCAACAAGCGCTCAGACGATGATGACAGCCCGAGTCTTATTTTTTTGA
CTGAAGAAGTCGCCCTGAAGGAGCCTTCAGATGATATATAATGCTTCTGGCTTTTCAATTGAATAAATAATACGTTTACCTGCAAC
AGCAACCATG

//

Halibut Hb29

TTATGAAGTTCACTGCCACCTTCTGCTGTTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTGGGAAATTTGGATGGGG
CCCCATATCAGCGGTAGAGTCACGGAATTAATTTGCTTTTTCATTGCAATATTTTAAATATTGCATAGCTGGAAAATCACGAAAT
AAGTAGTCGATATATTTGGCCAAATAGAATCACTTTGATTTCATAATAATCAAAAATAACAATCAAAAAGGCCCTTTGATTAGCATG
TTCTTCAATAAAATGGACATTGAAGTTTATTTTGATGCTCACATGCACCGACCTGCTGCGGCAACAATGAAATCAAATTTGCT
CAGAATTTAAAGTACATTTTCTAGGTGATTTAATCTTTCCATTAACTTGATTGTTTTTTATAAATATAGAATAACTGGATCTTTA
TGCCAAAATAATAAAACACACATTTCTGATTTTACAGTCAAGATTGAACACTACTTAAAAGTAATATAAAAACATCATCTGTATGTA
TAATTGTTTTAACTGTTTAAACAAAGTCCAAATAATTGTGTTATGGAATGTATTAATTGTTCATTTAATATCATTTGCTTGAATTCAT
CACCATGTGTTTTTTGTTGTTTTTACACAGGTGAAAAGAGGCCCTTGCAAGTAAGGACTTCTACCATCACTACTTTGTAATTTTGA
TAGTATATCATCTAGTACTGTTATTGACAACTTCTCTGTCTCGCTGACTCTCTCCATCAGGATGAACTCAGAGCGTCGAGTTAC
GACGAGCGGCAGCAGCAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

Halibut HbSc1A13

ATGAAGTTCACTGCCACCTTCTGCTGTTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTGGGAAATTTGGATCGTGCG
CCCTATCGGAGGTGAAAAGAGGCCCTTGACAGATGAATCAGAGCGTCGAGTTACGACGAGCGGCAGCAGCAGCAGCAGGAGCTCG
ACAAGCGCGCAGTCGATGAAA

//

Halibut HbSc1A24

ATGAAGTTCACTGCCACCTTCTGCTGTTGTTTCATGGTCTGCTCATAGCTGAACCTGGAGAGAGTCTTTTGGAAAGTTCTCAA
GAAAGTTGTCATGCTGGCAGCTCAATTGGCGAGACAGCCTTGATGTCGCGCAGAGCATCACGGGCTTCATGCGCATCACGGGT
GTCACGGGCGCTCACGGGGGTACAGGCGTCAAGGGGGTCAAGGCGTCAAGGCGTTCGCGGTTACGACGAGCAGCAGCAGGAGGAG
CTCGACAAGCGCGCATTCGATGA

//

Halibut HbSc1B34

TATGAAGTTCACTGCCACCTTCTGCTGTTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTGGGAAATTTGGATGGGGC
CCCATATCAGCGGTAGAAAGAGGCCCTTGACATGAATCAGAGCGTCGAGTTACGACGAGCGGCAGCAGCAGCAGCAGGAGCTC
GACAAGCGCGCAGTCGATGAAA

//

Halibut Hb17

ATGAAGTTCACTGCCACCTTCTGCTGTTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGTGTTTTTTGGGATTGCTTTTTCA
CGGGGTCACCATGTTAGGTCACGGAAGTAATTCGATTTTTCATGGCAATATTTTAAAGATAACACACCATATGAGTAGTCGAT
ATATTTGGCCAAATAGAATCACTTTGATTTCATAATAATCAAAAATAACAATCTCTAGGCGATTTAATATTGCAATTAATTGGATT
TGTTTTTAAAAATATAGAATAACTGGATCTTTATGGTAAAATAATTAACATACATTCTGATTTTACCAGTCAAGATTGAACACTA
CTTAGAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTTAAACGAATAGTCCAAATAATTGTGTTATGGAATGTA
TTAATTGTCTATTAAATATCATTTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACACAGTTGGAAGTTGATCCATGGGT
AAGGTTCTTACCATTCTACTGTGTTATTTAATAGTATTCATCAGTACTATTATTGACAACTTCTCTGTCTCGCTGACTCT
CTCCATCAGACTCATCCATGGCGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

Witch Flounder GC1.2

GCCCACTTTGTATTTCGCAAGGTAAGAGCGATATATTTCAAATTCATTTCGGATGAGACCAAGCATTGTTGGGAAATGTGCTCAGCTTGT
TACTGTTTAAATGCAAATGTTAACAATATCTCTTTTCTGTGTTTTTGTAGAATGAAGTTCGCTGCCGCTTCTCATGATGTTTCAT
GGTCGTCCTCATGGCTGAACCCGGAGAGGGCTCGTTGGGGAACGTTCTTCAAACATATTTTCAAAGGTAGAGTCACAGAATTAATTT
GCTTTTACATTGCAAATATTTTTCATATAACATAGCTGGAAAATCACAAAATAAGGGCTTGATATATTTGGCAAAGTAGAATCCC
TTTGATTTCAATAATAATCAAATAAAAAATCAGAAAGGCCCTTGATTAGCATGTTCTTCAATAAAATGGACATTGTAGTTTATTT
TGATTCTCAAATGCACCAACCTGCTGCGGCAACAATTGAAATCAAATTTGTCTCGAAACATTTAAAGTACATTTTTCGAGGCAAT
TTAATCTTTTCCCTTGATCGAATTCGTTTTTAAAAATATAGAATAACTGGATCTTTATGCTAAAAATAATAATCATACATTCTGATT
TTACCAGTCAAGATTGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATGTATAATGTTTAACTTTTAACTAATAGTCCT
AATAATTGTGTTATGGAATGTATTCTATTGTCTATTAAATATCATTTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACAC
AGCTGGAAGGTTTCATCCATGGGTAAGGACTTCTACCATCATTACTGTGTATTTTAAATAGTATTATCATCAGTACTGTTATTGATA
ACTTCTCTGTCTCGCTGACTCTCTCCATCAGTGCATCCAGGCACACAATGACGGCGAGCAGCAGGATCTCGACAAGCGCTCAGT
GGATGATGAGCCAGTGTATTGTTTTGAATGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

Witch Flounder GC1.3

GCCCACTTTGTATTTCGCAAGGTAAGAGCAATATATTTCAAATTCATTTAGACGAGACCAAGCATTGTTGGGATCTGTGCTCAACTTGT
AACTGTATAATGCAAATGTTAACAATATCTCTTTTCTGTGTTTTTGTAGAATGAAGTTCGCTGCCGCTTCTCATGATGTTTCAT
GGTCGTCCTCATGGCTGAACCCGGAGAGGGTCTTGGAATACCTGCTTGAATAGGATCTATCATGGTAGAGTCACAGAGTTAATTT
GCTTTTACATTGCAAATATTTTAAATATAACATGGCTGGAAAATCACAAAATGAGTACTCGATATATTTGGCAAAGTAGAATCCC
TTTGATTTCAATAATAATCAAACACCAATCAAAGGCCATTGATTAGCATGTTCTTCAATGAAATGGACATTGTAGTTTATTT
TGATTCTGACATGCACCAACTGCTGCGGCAACAATTGAATTCAAATTTGTCTCAGAAAAATTTAAAGTACATTTTCTTTCCATT
AGTCGGATTGTTTTTAAAAAATACAGAATAACTGGATCTTTATGCTAAAAATAATAATCATACATTCTGATTTTACCAGTCAAGAT
TGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATTGATAATTGTTTAACTTTTAACTAATAGTCCATAATTTGTGTTAT
GGAAATGTATTCTATTGTCTATTAAATATCATTTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACACAGCTCTACTGAGGA
TCAATCGGTAAGGACTTCTACCATCATTACTGTGTAATTTAATAGTATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTT
GCTGGCTCTCTCCATCAGCCAAATGGTGTATTATCGTCGGCACTGGCAGCGTGACGTCGAGCAGCAGGCTCTCGACAAGCGCTCAG
TGGAGGACCAGCCAGTTCTATTGCTTCTGCTGGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

Witch Flounder GC1.4

GCCCACTTTGTATTTCGCAAGGTAAGAGCAATATATTTCAAATTCATTTAGACGAGACCAAGCATTGTTGGGATCTGTGCTCAACTTGT
AACTGTATAATGCAAATGTTAACAATATCTCTTCTGTGTTTTTGTAGAATGAAGTTCGCTGCCGCTTCTCATGATGTTTCAT
GGTCGTCCTCATGGCTGAACCCGGAGAGGGTCTTGGAATGCTGCTTGAATAGGATCTATCATGGTAGAGTCACAGAGTTAATTT
GCTTTTACATTGCAAATATTTTAAATATAACATGGCTGGAAAATCACAAAATGAGTACTCGATATATTTGGCAAAGTAGAATCCC
TTTGATTTCAATAATAATCAAACACCAATCAAAGGCCATTGATTAGCATGTTCTTCAATGAAATGGACATTGTAGTTTATTT
TGATTCTGACATGCACCAACTGCTGCGGCAACAATTGAATTCAAATTTGTCTCAGAAAAATTTAAAGTACATTTTCTTTCCATT
AATCGGATTGTTTTTAAAAAATACAGAATAACTGGATCTTTATGCTAAAAATAATAATCATACATTCTGATTTTACCAGTCAAGAT
TGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATTGATAATTGTTTAACTTTTAACTAATAGTCCATAATTTGTGTTAT
GGAAATGTATTCTATTGTCTATTAAATATCATTTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACACAGCTCTACTGAGGA
TCAATCGGTAAGGACTTCTACCATCATTACTGTGTAATTTAATAGTATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTT
GCTGACTCTCTCCATCAGCCAAATGGTGTATTATCGTAGGCATGGCAGCGTGACGTCGAGCAGCAGGCTCTCGACAAGCGCTCAG
TGGAGGACCAGCCAGTTCTATTGCTTCTGCTGGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

Witch Flounder GcSc4B35

ATGAAGTTCATGCCACCTTCTGGTGTGTTTCATGGTCTGCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGTTTAC
TAAAGGTGCCAAGCACCTTGGCCAGGCGGCCATTACGGTTTGGCCTCTTGCGAAGAGCAGCAAGAGCTCGACAAGCGCTCAGAGG
ATGACGAGCCAGTGTATTGTTTTTGAA

//

Witch Flounder GC3.6

ATGAAGTTCATGCCACCTTCTGGTGTGTTTCATGGTCTGCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGCTCCG
TAAAGGTAGAGTCATGGATTAAATTTGCTTTTACATTGCAAATACTTTAAATATAACATAGTTGGAAAATCACAAAATAAGTAGT
CGATATATTTGGCCATATAGAATCACTTTGATTTCAATAATAATCAAACAAACATCAAAGGCCATTGATTAGCATGTTCTTCTC
ACTAAAATGGACATTGTCTATTATTTGATTCTCAGGACCAACCTGCTGCGGCAACAATTGAAATCAAATTTGTCTCAGAAGA
ATTCAAAGTACATTGTTCTAGGCGATTAAATCTTTCCATTCTCGGATTTGTTTTTAAAAATATAGAATAACTGGATCTCTATGTT
AAAATAATAAAACACACATTCTGATTTTACCTGTCAAGATTGAACAGCACTTAAAGTATGTATAAAACATCATCTGTATGTATAA
TTGTTTAACTGTCAACTAATAGTCCAAATAATGTGTTATGGAATGTATTCTATTGTATATAATATCATTTGCTTGAATTTATCA
CCATGTGTTTTTGTGTTTTTACACAGGTGCCAAGCACCTTGGCCAGGCGGCCATTAAAGTAAGGACTTCTACCATCATTACTGTG
TAATTTTAAAGTATTATCATCAGTACTGTTATTGACAACCTACTCTGTCTCTGTGACTCTCTCCAGGGGTTTGGCCTCTTGGCAA
GAGCAGCAGGAGCTCGACAAGCGCTCAATGGATGACGAGCCAGTGTATTGTTTTTGAAGTGTGCTTGAAGGAGCCTTC

AG

//

Witch Flounder GC2.2

GCCCACTTTGTATTTCGCAAGGTAAGAGCGATATATTTCAAACCTCATATAGACGAGACCAAGCATTGTTGGGAAATGTGCTCAGCTTGT
TACTGTATAATGCAAATGTTAACAATGTTTTTGTCTGTGTTTTTGCAGAATGAAGCTCGCTGCTGCCCTTCTGGTGTGTTTCAT
GGTCGTCCTCATGGCTGAACATGGAGAGGGTTTTTGGGATTCTATATGAAGCCTGGTAGAGTCAAGGAATTAATTCGATTTTAAAC

ATGGCAAATATTTTACTATAACATACCATATGAGTAGTCGATTAAATTAATTGGATTGTTTTTAAAAATATAGAATAATTGGATCT
TTATGCTAAAATAAATAACATACATTCTGATTTTACCAGTTAAGATTGAACGCTACTTAAAAGTATGTATAAAACATCATCTGTA
CATATAATTGTTTAACTGTTAAACCAATAGTCCAAATAATTGTTGTTGGAAATGTATTAATTGTCATTTAATATCATTTGCTTGAA
TTTGTACCATGTGTTGTTGTTGTTTACACAGGTAGAAAGATTTCCTATGGGTAAGGACCTTACCATCATTTACTGTGTATTT
TTAGCAGTATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTCGCTGACTCTCTACAGGTACATCAGAAGTCTTATGGTTA
CGACGAGCAGCAGGAGGTCGACAAGCGCTCAGTCGATGACAACCCAGTGGCATTGCTTCTGACTGAAGAAGTCGCCCTGAAGGAG
CCTTCAGA

//

Witch Flounder GcSc4B28

ATGAAGTTCAGTCCACCTTCCTGGTGTGTTTCATGGTCGTCCTCATGGCTGAACCTGGCGAGGGTTATTGGCGCTTCGCAACCA
CCGTGGTGAAAGGTTATCCAGAGGCATTTCGCTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGTGGATGACGAGCCAGTT
CTATTGCTTTTGA

//

Witch Flounder GC3.7

ATGAAGTTCAGTCCACCTTCCTCGTGTGTTTCATCGTCATGTTTGAACCTGGAGAGTGTGTTTTGGAATGCTTTTTACCGGGTCC
ACCATGTCGGGTACGGAAGTAGTTCGATTTTACATGGCAAATATTTAAATGAAACATACCATATGAGTAGTCGATATATTTGG
CCAAGTAGAATCATTGACTTCAATAATAATCAAAAACATAATCAAAAAGCCCATTTGATTAGCATGTTCCCTCAATGAAATGGAC
ATTGAGGTTTATTTTGATTCTCACAGGCACCAACCTGCTGCGCAACAATTGCATTCAAATTTGCCAAAGAACTTAATTAACA
TTTTCTGGCGATTAAATCTTGCATAAATTGGATTGTTTTTAAAAATATAGAATAACTGGATCTTTATGCTCAAATAATTAATCA
TACATTTCTATTTTATCAGTCAAGATTGAACGCTACTTAAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTTTTA
ACTAAAAGTCTAATAATTGTTTATGGAAATGTATTAATTGTCATTTAATATCATTTCCCTGAATTTATCACCATGTGTTTTGTT
TTGGTTTTTACACAGCTGGAAGGTTGATCCATAGGTAAAGGACTTCTACCATCATTTACTGTATAATGTTAATAATAGCATTATCATC
AGTACTGTTATTGATAACTTCTCTGCTCGCTGACTCTCCATCAGATTTCATCAAACGTCACGGGTGACGTCGAGCAGCAGGAGC
TCGACAAGCGCTCAGTGGATGACGAGCCAGTTCTATTGCTTTTGCCTGAAGAAGTCGCCCTG

//

Witch Flounder GC3.1

ATGAAGTTCAGTCCACCTTCCTGGTGTGTTTCATGGTCGTCCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGAC
TGCGGTCCACAATGGTAAAGTCAAGGAATTAATTCGATTTTACGTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATA
TATTGACCAAGTAGAATCATTTTGACTTCAATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAATTGGATT
GTTTTTAAAAATATAGAATAACTGGATCTTAATGCTAAAATAATTAACATACATTCTGATATTACCAGTCAAGATTGAACGCTAC
TTAAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTGCTGCTAATAGTCTTAATAATTGTTTATGGAAATGTAT
TCATTGTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTTACACAGCTGGAAGGTTGATCCATGGTA
AGGACTTCTACCATCATTTACTGTATAATTAAAGAGCATTATCATCAGTACTGTTATTGATAACTTCTCTGCTCGCTGACTCTC
TCCATCAGACTACTCGGCTTTTCATCATGGGCTCCCGGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGT
GGATGAGGAGCCAGTTCTATTGCTTTTGAAGTGAAGAAGTCGCCCTTGAAGGAGCCTTCAG

//

Witch Flounder GC4.1

ATGAAGTTCAGTCCACCTTCCTGGTGTGTTTCATGGTCGTCCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGAC
TGCGGTCCACAATGGTAAAGTCAAGGAATTAATTCGATTTTACGTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATA
TATTGACCAAGTAGAATCATTTTGATTTCATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAATTGGATT
GTTTTTAAAAATATAGAATAACTGGATCTTAATGCTAAAATAATTAACATACATTCTGATATTACCAGTCAAGATTGAACGCTAC
TTAAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTGCTGCTAATAGTCTTAATAATTGTTTATGGAAATGTAT
TCATTGTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTTACACAGTTGGAAGGTTGGTCCATGGTA
AGGACTTCTACCATCATTTACTGTATAATTAAAGAGCATTATCATCAGTACTGTTATTGATAACTTCTCTGCTCGCTGACTCTC
TCCATCAGACTACTCGGCTTTTCATCATGGGCTCCCGGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGT
GGATGAGGAGCCAGTGTCTATTGTTTTTGAATGAAGAAGTCGCCCTTGAAGGAGCCTTCAG

//

Witch Flounder GC4.4

ATGAAGTTCAGTCCACCTTCCTGGTGTGTTTCATGGTCGTCCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGAC
TGCGGTCCACAATGGTAAAGTCAAGGAATTAATTCGATTTTACGTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATA
TATTGACCAAGTAGAATCATTTTGATTTCATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAATTGGATT
GTTTTTAAAAATATAGAATAACTGGATCTTAATGCTAAAATAATTAACATACATTCTGATATTACCAGTCAAGATTGAACGCTAC
TTAAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTGCTGCTAATAGTCTTAATAATTGTTTATGGAAATGTAT
TCATTGTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTTACACAGTTGGAAGGTTGGTCCATGGTA
AGGACTTCTACCATCATTTACTGTATAATTAAAGAGCATTATCATCAGTACTGTTATTGATAACTTCTCTGCTCGCTGACTCTC
TCCATCAGACTACTCGGCTTTTCATCATGGGCTCCCGGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGT
GGATGAGGAGCCAGTGTCTATTGTTTTTGAATGAAGAAGTCGCCCTTGAAGGAGCCTTCAG

//

Petrale sole 02A(3)

ATGAAGTTCAGTCCACCTTCCTCGTGTGTTTCATGGTCATCGTCATGTTTGAACCTGGAGAGTGTGTTTTTGGAAATGCGTTTTCA
CGGGGTCCACCATGGTAGGGTCACAAAAGTGATTGATTATPACATGCCAAATATGTTAATGAAACATACCATATGAGCAGTCGTA
TTATTGGACAAGTAGAATCATTGATTTCATAGTAATTAATAAACAATCAAAAAGGCTTTGATTAGCATGTTCCCTTCAATG
AAATGGACATTGAGGTTTATTTGATTCTCACTGCATCGACCTGCTGCGCAACTATTGAATCAAAATTTGCCAGAAGAACT

AAATTAACATTTTCTAGGCCATCTAATCTTTGCATGAATTGGATTGGCTTTCAAAAATATAGAATAACTGGATATTTATGCTAAAA
TAATAAAAACACACATTTCTGATTTTACCAGTCAAGATTGAACACTACTTAAAAGTACGTATAAAAACATCATCTGTATGTATAATTG
TTTGACTTTTAAACAAATAGTCAAAATGATTGTTATGGAAATGCATTAAATGTTCATTTAATATCATTTACTTGAATTTATCACCATG
TGTTTGTGTTTTTTAGCAGGTGGAGGTTTTCTCAATGCGCAAGGACTTCTACCATCAATTACTGTGAATTTAATAGTATTATC
ATCAGTACTCTTATGACAACGTCTCTTGTCTCGTGCATCTCTCTATCAGATTAAACCCAGGGTATCGCGGTACGACGAGCAGC
AGGAGCTCGACAGCGCGCAGTCGATGA

//

Petrale sole 02B

ATGAAGTTCACATGCCACCTTCTCTGGTGTGTCTTGGTCTCTCATGGCTGAACCTGGAGAGGGTTTCTTTGGAGCCCTTCTCAA
AGGTAGAGTCAACGGAATTAATTTGATTGTACATGGCAAATAATTTTGTATAACATATCATATGAGCAGTCGATGATTTTGACCAA
GAAGAATCATTTTGAATTTCAATAATAATCAAATAACAATCTCTTGGAGATTATATATTTGCAATAATTGGATTTTATAAAATATA
GAACAACCTGGATCTTAATGCTAAATAATTAACATACATCTGATTTTACCAGTCAAAATTAACCACTACTTTAAAGTATGTATA
AAACATCATCTGTATGTTAATTGTTTAACTTTTAAACAAATAGTCCAAATAATTGTGTAATGGAAATGTATTCAATGTCTATATAAT
ATAGTTTGCTTGACTTTATCACCGTGTGTTTTGTTTGTTTTTTTCACAGGTGCCCAGGCGCTCCATGGGTAGGACTTCTACCATC
ATGACTGTGTAAGTTTAATAATATTATCATCAGTACTGTATTAAACGACTTCTCTTGTCTCGCTGACTCTCTCCATCAGAATCATC
CACAATGCTCGTCAAGGTTACGACGAGCAGCAGGAACCTCAACAAGCGCGCAGTCGATGA

//

Petrale sole PL1/2/2.1

GCCCCCTTTGTATTTCGCAAGGTAAGATCAATATTTTTCAAATTCATTTAGACGAGACCAACCGTTTGGCAATGTGCTCAGCTTGT
TATTGTATAATAACAAAGTTAAGCATCTTTATTTTTCTGTTTTTTTGTAGAATGAAGTTCACCTGCCACCTTCTCGATGTTGTTTAT
CTTCGCTCTCATGTTGAAGTCTGGAGAGTGTGGTTGGAAAGATTGGTTTCGTAAGGCTAAGAAAAGGTAGAATCAGGGAATTAATTA
GCTTTTTACATTGCAAAATAGATTTTTTATAACAGCTGGAATCACAATAAATAAGTGTATATTTGGCCAATTAGAATCACTT
TAATTTCAATAATAATCTAAATAACAACCTAAAGGCCCTTTGATTAGCATGTTCTTCAATGAAAAGGACATTTGAGGTTTTATTTTG
ATTCCTCACATGCACCGACCTGTGCGGCAACAATTGAATTCAGATTGTGCCAGAAGAATTCAAAGTACATTTTTCCAGGCGATTAA
ATCTTTCCATTACTCGGATTTAAAAATAAATAAATAGATAAAGTGTGATAAAACATCATCTGTATGATAATTGTTTAACTGTTAATAGTCTTAATAATT
CAAGTCAAGATTGAACACTATTAAAAAGTGTGATAAAACATCATCTGTATGATAATTGTTTAACTGTTAATAGTCTTAATAATT
GTGTTATGGAATGTATTAAATTTACATTAAATATCATTTGCTTGAGTTTACCATCATGTGTTTTGTTTGTTTTTTACACAGTTGGC
AAGACTGTTGGCGGCTTTGGCCCTTAAGTAAAGAACTTCTACCATCATTTACTGTATAATTTTGATAGTATTATCACCAGTACTGTTAT
TAACACTTCTCTTGTCTCGCTGACTCTCTCCATCCGACTCATCCGAGTCATTACCTTGGCGAGCAGCAGGAGCTTGCCAAGCGC
GCAGTCGATGACGACCCAGTGTTATTGTCTTTGACTGAAGAAGTCGCCTTGAAGGAGCCTTCAG

//

English sole 05A

ATGAAGTTCACATGCCACCTTCTCATGATTTTAATCTTCCTCTCATGGTGAACCTGGAGAGTGTGGTATTAGGAAATGGTTTAA
AAAGGCTGCTCAGCGTAAAGTACGGAATTAATTTGCTTTTTGCTTTTCAAAATATTTTTTATAGCAGCTGGAAAATCACAATAAT
AAATAGTCGATGTATTGGCCAATTAGAATCACTTTGATTTCAAATAATAATCTAAATAGCAACCTAAAGGCCCTTTGATTAGCAT
GTTCTCTCAATGAAATGGATGTTGAGGTTTATTTTGATTCTCATGTCACCGACCTGCTGCGGCAACAATTGAATTCAAATTTGTC
CCAAAGGAATTCAAAGTAACTTTTCTAGATGATTTAATCTTTCCATAACTCGGCTTTGTTTTTAAAAATATATAAATACTCAATC
ACTATGATAAAATAATAACACATACATCTGATTTATACAGCAAGATTGAAAACCTTCTTAAAGTATGTATAAAACATCATCTG
TTTGTATAATTGTTTATCATTTTCAAAAAAGTCCAACATAATTGTGTTATGGAATTGTATAAATGTTCATTAAATATAATTTTTG
AGTTTATCAATATGTGTTTTGTTTGTTTTACACAGTTGCAAGGAAGTTGGCAAGGTGGCCCTTAAGTAAAGGACTTCTACCATTA
TTACTGTATAATTGTTGATGATTATCACCCGTACTGTTATTGACAACCTCTCTTTTCTGCTGACTCTCTCCATCTGACTCATCT
GCAGTGCTTGCCTTGACAAGCAGCAGCAGCTCGACAAGCGCGCAGTCGATGA

//

English sole PL1/2/5

GCCCCCTTTGTATTTCGCAAGGTAATATCGATATTTTTCAAATTCATTTAGACGAGACCAAGCATTTGGGAAATGTGCTAAGGTTGT
TACTGTATAATGCAAAATTAATGATCTTTATTTTTCTGTTTTTTTTTGGCAGAATGAAGTTCACCTGCCACCTTCTCATGATTTTAA
TCTTCGCTCTCATGGTGAACCTGGAGAGTGTGGTTTGAAGAAATGGTTTAAAAAGGCTGTTACGGTAGAGTCACGGAATTAAT
TGCTTTTTGCTTTTACAAATATTTTTTATAGCAGCTGGAAAATCACAATAAATAAGTGTGATGATTTTGGCCAATTAGAATCACT
TTGATTTCAATAATAATCTAAATAGCAACCTAAAGGCCCTTTGATTAGCATGTTCTTCAATGAAATGGATGTTGAGGTTTATTTT
GATTCTCATGTCACCGACCTGCTGCGGCAACAATTGAATTTCCAATTTGTTCCCAAAGGAATTCAAAGTAAACCTTTCTAGGCGATT
TAATCTTTCCATAACTCGGCTTTGTTTTTAAAAATATATAAATACTCAATCCCTATGATAAAATAATAACACATACATTCTGATTT
ATACAAGACAAGATTGAAAACCTTCTGAAAGTATGTATCAACATCATCTGTTGTATAATTGTTTAAACAGTTCACAAAAGTCCA
ACTAATGTGTTATGGAATGTATAAATGTTCATTTAATATAATTTTTTGGAGTTTATCAATATGTGTTTTGTTTGTTTTTACACA
GTTGGCAAGAAAGTTGGCAAGGTGGCCCTTAAGTAAGGACTTCAACATTTACTGTGTAATTTTGATAGTATTATCACCAGTAC
TGTTATTGACAACCTCTCTTCTGCTGACTCTCTCCATCCGACTCATCTGAGTGCTTACCTTGGCGAGCAGCAGCTCGAC
AAGCGTCAGTCGATGAAGAGCCAGTGTTATTGCTTTTGAAGTGAAGAAGTCGCCTTGAAGGAGCCTTCAG

//

Starry flounder 09A

ATGAAGTTCACATGCCACCTTCTCATGATGTTTATCTTCTGCTCTCATGGTTGAACCTGGAGAGTGTGGTTGGAGGAAATGGATTAA
AAAGGCTACTCAGGTAAAGTCAAGGAATTAATTCGTTTTTTGCTTTTGCAAATATTTTTTTTATAACAGCTGGAAAGTCAAAAA
TAAATAGTCAATATATTTGGCCAATTAGAATCACTTTGAGTTCAATAATAATCTAAATAACAACCAAAAGGCCCTTCTCTTAATG
AAATGTACGTTGAAGTTTATTTGAATCTCACATGCACCGACCTGCTGCGGCAACAATTGAATTCAAATTTCTCCAGAGGAATTC
AAAGTAAATTTTTCTAGGCGATTAAATCTTTCCATTACTCTGATTTGTTTTAAATATATAGAATGACTCAATTGCTATGATAAAT

AATAAGCCATACATTCTGATTTTACAAGACAAGATTGAAAACCTTCTTAAAAGTACGTATAAAACATCATCTGTATTATAATTGT
TTAACATTTAACAAATGTCTACTAAATTGTGTATGGAATGTATAAAATGTTCATTTAATATCATTGTCTGAGTTTATCATTAT
TTGTTTTTGTGTTTTTACACAGTTGGCAAGCATATTGGCAAGGCGGCCCTTGAGTAAGAACTTCTACCATCATTACTGTATAAT
TTTGATAGTATTATCACCAGTACTGTTATTGACAACTTCTCTGTCTGATGACTCTGTTTCATCCAACCTCATCTGCAGTGCTTACA
TTGGCGGAAGCAAGAACTCGACAAGCGCGCAGTCGATGA

//

Greenland halibut 12B

ATGAAGTTCACTGCCACCTTCCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTTTTCGGATTGCTTTTTTCA
CGGGATCCACCATGGTAGGGTTCACGGAATTAATTAGATGTTTACATGGCAAATATTTTAAGATAACACACCATATGAGTAGTCGAT
ATATTTGACCAATTAGAATCACTTTAATTTCAATAATAATCACAATAACAATCTCTAGGCCATTTAATCTTTCCATTAAATCGGATT
TGTTTTTTTAAATATAGAATAACTGGATCTTTATGCTAAAATAATGAAACATACATTCTGATTTTACCAGTCAAGATTGAACGTTA
CTTAAAGTATGTTTAAACATCATCTGTATGTATAATTGTTTAGCTGTAAACAAATAGTCCAAATAATTGTGTTATGGAATGTA
TTAATTGTTCATATAATAATTGTCTTGAATTTATCACCATTGTTTTTGTGTTTTTTTAAACACAGCTGGAAGTTGATCCATGG
GTAAGGACTTCTACCATCATCTACTGTGTATTTTAAATAGTATTATCATCAGTACTGTTATTAAACAACCTTCTCTCTATCGCTGACT
CTCTCCATCAGACTCATCCATCATGTTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

Pacific halibut 15A

ATGAAGTTCACTGCCACCTTCCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTTTGGGAAATTGGATGGGGCC
CCATATCAGCGGTAGAGTTCACGGAATTAATTGCTTTTTCCATTGCAAATATTTTAATATTGCATAGCTGGAAAATCAGAAATAA
GTAGTCGATATATTTGGCCAAATAGAATAACTTTGATTTCAATAATAATCAAAATTAACAATCAAAAAGGCCCTTTGATTAGCATGTT
CCTTCAATAAAATGGACATTGAAGTTTATTTTGATGCTCACAATGACCGACCTGCTGCGGCAACAATTGAAATCAAAATTTGTCTCA
GAATTTAAAGTACATTTTTCTAGGTGATTTAATCTTTCCATTCACTGATTTATTTATAAATATAGAATAACTGGATCTTTCTGC
TAAATAATAAAACACACATTCTGATTTTACCAGTCAAGATTGAACACTACTTAAAAGTATGTATAAAACATCATCTGTATGTATA
ATTGTTTAACTGTTTAAACAATAGTCCAAATAATTGTGTTAAGGAAATGTATTAAATGTTCATTTAATATCATTGTCTGAATTTATCA
CCATGAGTTTTTTGTTTTTTTACACAGGTAGAAAGAAGGCCCTTGCAAGTAAAGGACTTCTACCATCATTACTTTGTAAATTTTATA
GTATTATCATCAGTACTGTTATTGACAACCTTCTCTGTCTCGCTGACTCTCTCCATCAGGATGAACCTCAGAGCGTCCGAGTTACGA
CGAGTAGCAGCAGAAGCTCGACAAGCGCGCAGTCGATGA

//

Pacific halibut 15B

ATGAAGTTCACTGCCACCTTCCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGAGGTGTTTTTGGGATTGCTTTTTTCA
CGGGTCCACCATGGTAGGGTTCACGGAAGTAAATCGATTTTTACATGGCAAATATTTTAAGATAACACACCATATGAGTAGTCGAT
ATATTTGATATATTAGAATCACTTTGATTTCAATAATAATCAAAATAACAATCTCTAGGCGATTTAATATTGCTTTTGTGCTTTAC
TGTTTTTAAAAATATAGAATAACTGGATCTTTATGTTAAAATAAATTAAACATACATTCTGATTTTACCAGTCAAGATTGAACACTA
CTTAGAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTAACTAATAGTCCAAATAATTGTGTTATGGAATGTA
TTAATTGTTCATTAAATATCATTGTCTGAATTTATCACCATTGTTTTTGTGTTTTTTTACACAGTTGGAAATTTGATCCATGGGT
AAGGACTTCTACCATCATTACTGTGTATTTTTAATAGTATTATCATCAGTACTGTTATTGACAACCTTCTCTGTCTCGCTGACTCT
CTCCATCAGACTCATCCATCAGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

C-0 sole PL1/2/6

GCCCCCTTTGTATTGCAAGGTAATATOGATATTTTTCAAACCTCATTAGACGAGACCAGGCATTTGGGAAACGTGCTAAGGTTGTTACTG
TATAATGCAAAATTAATGATCTTTATTTTTCTGTTTTTTTTTGCAAGTGAAGTTCACTGCCACCTTCCTCATGATTTTAATCTTCGTCTCT
CATGTTGCAACCTGGAGAGTGTGGTATTAGGAAATGGTTTAAAAGGCTGCTCACGGTAAAGTCAAGGAATTAATTTGCTTTTTGCTTTAC
AAATATTTTTTACAGCAGCTGGAAAATCACAATAATAAATAGTCTGATGTTTTGGCCAAATAGAATCACTTTGATTTCAATAATAATCTA
AATAGCAACCTAAAAGGCCCTTTGATTAGCATGTTCTTCAATGAAATGGGTGTTGAGGTTTATTTTGATTCTCATGTCACCGACCTGCTG
CGGCAACAATTGAATTCAAATTTGTCCCAAAGGAATTCAAAGTAAACCTTTCTAGGCGATTAAATCTTTCCATAACTCGGCTTTGTTTTTA
AAAATATATAATACTCAATCGCTATGATAAAATAATAACACATACATTCTGATTTATACAAGACAAGATTGAAAACCTTCTTGAAGTATG
TATCAACATCATCTGTTTATATAATTGTTTAAACATTTCAAAAAGTCCAACTAATTGTGTTATGGAATTTGATAAATTTGTCATTTAATA
TAATTTTTTTGAGTTTATCAATATGTGTTTTTGTGTTTTTACACAGTTGGCAAGAAAGTTGGCAAGGTGGCCCTTAAGTAAGGACTTCTA
CCATTATTACTGTATAATTTTGATAGTATTATCACCAGTACTGTTATTGACAACCTTCTCTTTTCTGCTGACTCTCTCCATCCGACTCATC
TGCAGTGCTTACCTTTGGCGAGCAGCAGCAGCTCGACAAGCGTGCAGTCGATGAAGAGCCAGTGTATTGCTTTTGACTGAAGGAGTCCGC
TTGAAGGAGCCTTC

//

Table 13

Clone Name	Signal peptide	Anionic propiece	Mature peptide
Hal7.5	MKTFSVAVVAVVLAFILESTAVPFSEVTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QSHLSLRWCNCCHN-KGCGFCCKF		
Sal4.1	MKTFSVAVVAVVLAFILESTAVPFSEVTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QSHLSLRWCNCCHN-KGCGFCCKF		
Sal1	MKAFSVAVV--LVIAFILESTAVPFSEVTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QIHLSLGLGCNCCHN-IGCGFCCKF		
Sal2	-----RTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QSHLSLRWCNCCHN-KGCGFCCKF		
Sal2.1	MKTFSVAVV--PVIACFILESTAVPFSEVTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QSHLSLRWCNCCHN-KGCGFCCKF		
Sal2.4	MKQFSVAVV--LVMACFILESTAVPFSEVTEEVESIDSPVGEHQPGTSMNLPMHFRFR-QIHLSLGLGCNCCHN-IGCGFCCKF		
WF1	MKAFSIAVAVTLVLAFCIQSSAVPFQVQELAEAGNDTPVAEHQVMSMESWMPNPNRQKR-----GFKCKFCGCGCRA-GVCGLCCKF		
JapF2.4	MKTFSVAVATLVLAFCIQSSAVPFQVQELAEAGNDTPVAEHQVMSMESWMPNPNRQKR-----GFKCKFCGCGCRA-GVCGLCCKF		
WF3a	MKTFSVAVTAVLVLFICIQSSASFPQAQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
WF3b	MKTFSVAVTAVLVLFICIQSSASFPQAQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----GFKCKFCGCGCRA-GVCGLCCKF		
WF4	MKTFSVAVTAVLVLFICIQSSATFPE-----MPYNRQKR-----GFKCKFCGCGCRA-GVCGLCCKF		
WFD1.1	MKTFSVAVTAVLVLFICIQSSASFPQAQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
WFD1.4	MKTCSVAVTAVLVLFICIQSSASFPQAQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP5.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP5.3	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP5.4	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP5.5	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP6.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP6.2	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP6.4	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
AP6.3	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Sal8.6	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal7.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal7.4	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal8.2	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal8.3	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal5.3	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
Hal1.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
WF2	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
YT11.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
YT12.1	MKTSSVAVTAVLVLFICIQSSATFPEVQLEEAHSNDNAAAEHQETPVDSDWMPNPNRQKR-----SFCKFCGCGCRA-GVCGLCCKF		
GC9.3	-----MPNNRQKR-----GSGCKFCGNCCHN-----GCGTCCCV		

Appendix I. Nucleotide sequences of pleurocidin-like genes and cDNAs referred to in Table 4.

NRC-01

ATGAAGTTCACCTGCCACCTTCCTCCTGTTGTTTCATCTTCGTCCTCATGGTTGATCTCGGAGAGGGTCGTCGTAAGAAAAAGGGGTCG
AAGAGAAAGGGGTCCAAGGGAAGGGGTCCAAGGGAAGGGCAGGTGGTTGAAAGGATTGGTAAAGGTAGAGTCACGGAATTAATT
TGCTTTTACATGCAAAATATTTTTCATATAACATTGCTGGAATAATCACAAAAATAAGTAGTCAATATATTTGGCCAAATAGAATCA
CTTTGATTTCAATAATAATCAAAATAACAACCTAAAAGGCCTTTGATTAGCATGTTCTTCAATGAAATGGACATGTAAATTTACTT
TGATTCTCACATGCTACGACCTGCTGCAGCAACATTGAAAAATAAATTTGTCCAGAGATTGTTAAAGTACATTGTTATAGGCGATT
TATCTTTCTATTACTCAGATATTTGTTCAAACCAATAGATAAATCTGATGTATGATAAATTGTTAACTGGTAACCTTATAGTCCTAATA
CCAGTCAAGATTGAAGCTGTTTTAAAAGTAAGTATGAAACATCCTCTGTATGTATAAATTGTTAACTGGTAACCTTATAGTCCTAATA
ATTGCGTTATGGAATGTATTAAATTGTCATTAAATATAAATTTGCTGGAATTTATCACTGTGTGTTTTGTTTGTGTTTTACACAGCTG
GCGGGATAATTATCGGGGGGGCCCTTGAGTAAGGACCTTACCATTACTGTGTAATATTTATAGTTATGATCAGTACAGTTATT
AACAACTTCTTCTGCTGCTGAACCTTCTCCATCAGTCACCTCGGGCAGGGGCAGGTGCAGGGGCCGATTACGACTACCAGGAGGG
GGAGGAGCTCAACAAGCGCGCAGTCGATGAA

//

NRC-02 and NRC-03

ATGAAGTTCACCTGCCACCTTCCTCCTGTTGTTTCATCTTCGTCCTCATGGTTGATCTCGGAGAGGGTCGTCGTAAGAGAAAGTGGTTG
AGAAGGATTGGTAAAGGTGTCAAGATAATTGGCGGGGGCGCCCTTGATCACCTCGGGCAGGGGCAGGTGCAGGGGCAGGATTACGAC
TACCAGGAGGGGCAGGAGCTCAACAAGCGCGCAGTCGATGAAA

//

NRC-04

GCCCACTTTGTATTTCGCAAGGTAATATTGATATTTTTCATATTCATTTAGACAAATGTGCTCAGCTTGTACTGTATAATGCAAAAG
TTAATGATCTTTATTTTCTGTTTTTTTTTTGTAGAATGAAGTTCACCTGCCACCTTCCTCATGATTGCCATCTTCGTCCTCATGGTTG
AACCTGGAGAGTGTGGCTGGGGAAGCTTTTTTAAAAGGCTGCTCACGGTAGAGTCACAGAATTAATTAGCTTTTTGCTTTGCAAAAT
ATTTTTTTTATAACAGCTGGAATAATCACAAAAATAAATAGTATATATTTGGCCAAATAAATCACTTTGATTTCAATAATAATCTA
AATAACCAACCTAAAAGGCCTTTGATTAGCATGTTCTTCAATGAAATGTACGTTGAGGTTTTATTTGATTTCTCAAGCACCACCC
TGCTGCGTCAACATTTGAATTTGCCAAAGGAATTCAAAGTAAATTTTTCTAGGCGATTAAATCTTTCCATTACTCTGAT
TTGTTTTTAAAATATAGATAAATCAATCTCTATGATAAAACAATTACACATACATTGAGTTTTATAGGACAAGATTGAAAACCTT
CTTACAGTATGTATAAAACATCATCTGTATGTATAAATTGTTTAAACATGTAACAACCTAGTCCTACTAATTGTGTTAAATTTGTCATT
AATATCAATTGCTTGAGTTTATCATTATGTGTTTTGTTTTTTTTTACACAGTTGGCAAGCATGTTGGCAAGGCGGCCCTTACGTAAG
GACTTCTACCATTTTACTGTATAAATTTGATAGTGTATCACCAGTACTGTTTTTGACAACTTCTCTATTCTGCTGACTCTCTCCA
TCCGACTCATCCGAGTCATTACCTTGGCGATAAGCAGGAGCTCAACAAGCGTGCAGTCGATGAAGACCCAAATGTTATTGTTTTTG
AATGAAGAAAT

//

NRC-05

ATGAAGTTCACCTGCCACCTTCCTGGTGCTGCTCCCTGGTGCCTTAATGGCTGAGCCTGGAGAGTGTCTTCTAGGAGCCCTTATCAAA
GGGGCCATCATGGTAGAGTCAAGGAATTAATTAGATTTTACATGTCAAATAATGTAGTAGAACATATATAAGTAGTCAATATATT
TGACCAAGTAGAATCATTTTGTATTCAATAATAATCAAAATAACAATCTCCAGGCGATTAAATATTTGCAATAATTGGATTTTATAG
AATACGGAACCACTGGATCTTAATGCTAAAATAATCCAACATACATTCTGATTTTGGCAGGCAAAATTAACACTACTTTAAAGTAT
GTATAAACAATAATCTGTATGTTTATAACAATACTCCAAAGCAATTTGTGTGATGGAAATGTATTATTGTCATTAAATATAAATTGCT
TGAGTTTATCATCTTGTGTTTTTGTGTTTTTTTACAGGTGGCAGGTTATCCATGGGTAAAGGACTTCTACCATCATGACTGTGTA
TTTTTAATATTATTATCATCAGTACTGTTATTGACAACTTCACTTGTCTGCTGACTCTCTCCATCAGAATGATCCAAACCATCAC
GGTTATGACGAGCAGCAGGAGCTCAACAAGCGCGCAGTCGATGAA

//

NRC-06

GCCCACTTTGTATTTCGCAAGGTAATATCAATATTTTTCAAATTCATTTAGACGAGACCAACCTTTGGGAAATCTGCTCAGCTTATT
ACTGTATAATGCAAAATGTTAATGATCTTTATTTTTCTGTTTTTTTTTTGTAGAATGAAGTTCACCTGCCACCTTCCTCATGATGTTCA
TCTTCGTCCTCATGGTTGAACCTGGAGAGTGTGGTTGGGGAAGCATTTTTTAAGCATGGTCGTCATGGTAAAGTCACGGAATTAATTA
GCTTTTAACTTTGCAAAATATTGTTTTTTTTTTTAAACAGCTGGAATCTCACAAAAATAAATAGCCGATATTTGGCCAAATTATAATC
ACTTTGATCTAAATAACAACCTAAAAGGCCTTTTGAATGATGATTTCTTCAATAAATAGATTGAACACTACTTAAAGGTATGTATAA
AACATCATCATGTGTTTTTGTGTTTTTTTACACAGCTGCCAAGCATATTGGCCATGCAGCCGTTAAGTAAGGACTTCTACCATTATT
ACTGTATAAATTTGATAGTATTATCACCAGTATTGTTATTGACAACTTCTCTTTTTCTGCTGATCCGACTCATCCGAGTCATTAC
CTTGGCGAGCAGCAAGATCTCGACAAGCGCGCAGTCGATGAAGACCCAAATGTTATTGTTTTTGAATGAAGAAAT

//

NRC-07

ATGAAGTTCACCTGCCACCTTCCTCATGATGTGCATCTTCGTCCTCATGGTTGAACCTGGAGAGTGTGCTTGGGGGAAATGGTTTAA
AAGGCCACACACGCTAGAGTCACAGAATTAATTAGCTTTTGTCTTTGCAAAATATTTTTTATAACAGCTGGAATAATCACAAAAATAA
ATAGTCTATATATTTGGCCAAATTAGAATCACTTTGCTTTCAATAAAATCTAAATAACAACCTAAAAGTCTTTGATTAGCATTTTC
CATCAATGAAATGGACGTTGAGGTTTTATTGATTCTCACATGCACCGACCTGCTATGTCAACAATTGAATACAATTTGTCCGAGA
GGAATCAAAGGAAATTTTTCTAGGCGATCTAATCTTTCCATTACTCGGATTTGTTTTAAATATATAGAATAAATCAATCTCTATG
ATAAATAATAACACATACGTAAAGATTTTCAAGACAAGATTGAAAACCTTCTAAAAGTACGTATAAACAATCATCTGTATTAT
AATTGTTTAAATTTAACAATAGCCCTACTAATTGTGTTATGGAATGTATAAATTTGTCATTAAACATAAATGTTTGTGATTATC
ATTATTGTTTTTGTGTTTTTTTACACAGTTGGCAAGCATTTGGCAAGGCGCCCTTACGTAAGGAGCTTCTACCATTACTGTA
TAATTTGATAGTATTATCACCAGTACTGTTATTGACAACTTCTCTTGTCTGCTGACTCTCTCCATCCGACTCATCCATAGTGCTT
ACCTTGGCGACAAGCAAGAACTCGACAAGCGCGCAGTCGATGAA

//

NRC-08

TAATAAACTAATGTGTAAAGTCTTCCACTTTTTTACTGTATTTACTTAAACAGAAATTAATCTCACGATTCTGGAGCTGCAGCC
ACTAAGTGTGCTTCATGAAGTGAATACAAATGTTCTAACACCACTCACCAATTAACGAGATCTACAAAGTGAGGAAGTGAG
AGGAGTCGTCCTGTGTTTTCAAATTTTTTGAATGATCTACCACTATGTGAGCTCCTCTGTTATAGCTCTAAATGTTACAAATGAA
TGTGAAGTCAGTTCTGTGTATATAAAGAGTTGCTCTGTAGAGCATACAACAGATTTACCTTTGAATCTCAAAACCTCACTTTGT
ATTCGACAGGTAAGATCGATATTTTTCAAACCTCATTTAGACGAGACCAAGTATTTGGGAAATGTGCTCAGCTTGTCAATGTATAATG
CAAATGTTTAAACATCGTTTGTCTTATGTTGTTGTTGATGAGTGAAGTTCGCTACTGCTTCTGATGTTGTCCATGGTCCCTC
ATGGCTGAACCTGGAGAGTGTGCTTCTACAGAGGACATCAAGTCTATCTCGGGTAGAGTCCAGGAATTAATTATTATCAATAAC
AATGAATAACAACCAAAAGGCCTCTGATTAGCATGTTCTTCAATGAAATGGTGGTTTTTATCTATTTTGAATCTCACATGCAAC
GACCTGCTGCGGCAACATTTGAAAATCAATCTTTTTTACAAATTCAAAGTACATTGATTTATTGATTTAATCTTAACATTAATC

11

//

11

SUBSTITUTE SHEET (RULE 26)

//

NRC-13

TTGCCCACTTTGTATTGCGAAGGTAAGATCAATATTTTCAAATTCATTTAGACGAGACCAACCATTTGGGAAATGTGCTCAGCTTG
TTACTGTATAATGCAAAAGTTAAGTATCTTTATTTTCTGTTTTTTTTGTAGAAATGAAGTTCACTGCCAATCTCCTCATGTTGTTTC
ATCTTCGTCCTCATGTTTGAACCTGGAGAGTGTGGTTGGCGAACATTGCTTAAAAAAGCTGGTCACGGAATTAATACGCTTTTTTACA
TTGCAAAATAGATTTTTTATAACAGCTGGAAAATGACAAAAATAAATAGTCGATATATTTGGCCAATTAGAATTATTTGATTCTCAAT
AATAATCTAAATAACAACCTAAAAGGCTTTTGATTAGCATGTTCTTCAATGAAATGGACATTGAGGTTTATTTTGATTCTCAATG
ACCGACCTGCTGCGGCAACAATTGAATTCAGATTTGTCCAGAGAATTCAAAGTAAATTTCCAGGGGATTAAATCTTTCCATTAC
TCGGATTAAAAAATAAATAAGATAAATGAATTCGCAATGAAAAATAATTACACATACTGTCTGATTTTACAAGTCAAGATTG
AACACTACTTAAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTAAACAAATAGTCCAAATAATTGTGTTATGGA
AATGTATTAATTGTCATTAAATATAAATTGCTTGAGTTTATCATCATGTTGTTTTTTTTTTTTTTTACACAGAGGTTAAGACTGTTG
GCAAGTTGGCCCTTAAGTAAGGACTTCTACCATCATTAAGTATGATAATTTTGATAGTATATCACCAGTACTGTAGTACTGACAACTT
CTCTCTCCACCACTCATCCGAGACATTACCTTGGCAAGCAGCGGAGCTCGACAAGCGCGCAATTGATGACGACCCAGTATTA
TTGTTTTTGACTGAAGAAGTTCGCTTGAAGGAGCCTTCAGAA

//

NRC-14

ATGAAGTTCACTGCCACCTTCTCATGATGTTTCAATGCTCGTCTCATGGCTGAACCCGAGAGGCTGGTTGGGGAAGTATTTTCAA
CATATTTTCAAAGCTGGAAGTTTCAATCATGTTGCGATCCAGGCACACAATGACGGCGAGGAGCAGGATCTGCACAAGCGCGCAGTC
GATGA

//

NRC-15

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCAATGCTCGTCTCATGGCTGAACCTGGAGAGGGTTTTTGGGGAAGCTTTTGA
TTGGGCATGATGGAATCGGGCTGCTCCATCAGCATTTGGGTGCTGACGAGCAGCAGGAGCTCGACGAGCGCTCAGAGGAGGACGAG
CCCAATGTTATGTTTTTGAATGAAGAAGTCGCAATGAAGGAGCCTTCAG

//

NRC-16 and NRC-17

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCAATGCTCGTCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGCTCCGT
AAAGGTAGAGTCATGGATTTAATTTGCTTTTTTACATTGCAAAATCTTTAATAACATAGTTGGAAAAACCAAAAAATAAGTAGTCG
ATATATTTGGCCATATAGAATCACTTTGATTTCAATAATAATCAAAACAATAATCAAAAAGCCATTGATTAGCATGTCCCTTCACT
AAAATGGACATTGTAATTTATTTTGATTCTCAGAGGCACCAACCTGCTGCGGCAACAATGAAATCAAATTTGTCTCAGAAGAATTC
AAAGTACATTGTTCTAGGCGATTAACTCTTCCATTTCATCGGATCTGTTTTTAAAAAATATAGAATAACTGGATCTCTATGTTAAAT
AATAAAACACACATTCTGATTTTACCTGTCAAGATTGAACACGACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTT
AACTGTCAACTAATAGTCCAAATAATTGTGTTATGGAATGTATTCAATTGTCATATAATATCAATTGCTTGAATTTATCACCATGTTG
TTTTTGTGTTTTTACACAGGTGCAAGCACCTTGGCCAGGCGGCCATTAAAGTAAGGACTTCTACCATCATTAAGTATGTAATTTTA
ACAGTATTATCATCAGTACTGTTATTGACAACTACTCTTGTCTCTGTTACTCTCTCCAGGGGTTTGGCCTCTTGGCAAGAGCAGCAG
GAGCTCGACAAGCGCTCAATGGATGACGAGCCAGTGTCTATGTTTTTGACTGAAGAAGTCGCTTGAAGGAGCCTTCA

//

NRC-18

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCAATGCTCGTCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGTTCACT
AAAGGTAGAGTCATGGATTTAATTTGCTTTTTTACATTGCAAAATCTTTAATAACATAGCTGGAAAAATCAAAAAATAAGTAGTCG
ATATATTTGGCCATATAGAATCACTTTGATTTCAATAATAATCAAAACAATAATCAAAAAGCCATTGATTAGCATGTCCCTTCACT
AAAATGGACATTGTAATTTATTTTGATTCTCAGAGGCACCAACCTGCTGTGGCAACAATGAAATCAAATTTGTCTCAGAAGAATTC
AAAGTACATTGTTCTAGGCGATTAACTCTTCCATTTCATCGGATTTGTTTTTAAAAAATATAGAATAACTGGATCTCTATGTTAAAT
AATAAAACACATTCTGATTTTATCTGTCAAGATTGAACACGACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAA
CTGTCAACTAATAGTCCAAATAATTGTGTTATGGAATGTATTCAATTGTCATATAATATCAATTGCTTGAATTTATCACCATGTTGTC
TTTTGTTGTTTTTACACAGGTGAAAGGTTATCCCAGAGGTAAGGACTTCTACCATCATTAAGTATGTAATTTTAAATAGTATTATCATC
AGTACTGTTATTGATAACTTCTCTTGTCTCGTGACTCTCTCCATCAGGCATTCTGTCGAGCAGCAGGAGCTCGACAAGCG
CTCAGTGGATGACGAGCCAGTCTATTGCTTTTGACTGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

NRC-19

TTATGAAGTTCACTGCCACCTTCTGGTGTGTTTCAATGCTCGTCTCATGGCTGAGCCTGGAGAGTGTTTTTTGGGATTGCTTTTTTC
ACGGGGTCCACCATGGTAGGTCACGGAATTAATTCGATTTTTTACATGGCAAATATTTAAGATAACACACCATATGAGTAGTCGAT
ATATTTGACCAATTAGAATCACTTTAATTTCAATAATAATCAAAATAACATCTCTAGGCCATTAACTCTTCCATTAAATCGGATTT
GTTTTTTAAATATAGAATAACTGGATCTCTATGTTAAATAATAAAACATACATCTGATTTTACCAGTCAAGATTGACGCTACT
TAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTAACTAATAGTCCAAATAATTGTGTAATGGAATGTATTA
ATTGTCATTTAATATCAATTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACACAGTTGGAAAGTGGATCCATGGGTAAAGG
ACTTCTACCATCATTAAGTATTTTAAATAGTATTATCATCAGTACTGTTATTGATATTTTCTCTTGTCTCGCTGACTCTCTCCA
TCAGACTCATCCATGGGCATCAGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTTCGATGAAA

//

NRC-20

TTATGAAGTTCACTGCCACCTTCTGGTGTGTTTCAATGCTCGTCTCATGGCTGAACCTGGAGAGGGTTTTTGGGAATTTCTTTTTTC
ACGGGGTCCACCATGGTAGGTCACGGAATTAATTCGATTTTTTACATGGCAAATATTTAAGATAACACACCATATGAGTAGTCGAT
ATATTTGACCAATTAGAATCACTTTAATTTCAATAATAATCAAAATAACATCTCTAGGCCATTAACTCTTCCATTAAATCGGATTT
GTTTTTTAAATATAGAATAACTGGATCTCTATGTTAAATAATAAAACATACATCTGATTTTACCAGTCAAGATTGAACACTACT
TAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTAACTAATAGTCCAAATAATTGTGTAATGGAATGTATTA
TTGTCATTTAATATCAATTGCTTGAATTTATCACCATGAGTTTTTGTGTTTTTACACAGGTAGAAAGAGGCCCTGCAGTAAGG
ACTTCTACCATCATTAAGTATTTTAAATAGTATTATCATCAGTACTGTTATTGATATTTTCTCTTGTCTCGCTGACTCTCTCCA
TCAGGATGAACCTCAGAGCGTCGAGTTACGACGAGCGGCAGCAGCAGCAGGAGCTCGACAAGCGCGCAGTTCGATGAAA

//

NRC-101

GCCCACTTTGTATTGCGAAGGTAAGATCGATATTTTCAAATTCATTTAGACGAGACCAAGCATTTGTTGAAATGTGATAAGCTTCT
AACTTTATAATGCAAAATGTTAAACAATCTTTTTGTTCTGTTGTTTTTGTAGGATGAAGTTGGCTGCCGCTTCTGGTGTGTTCTCTG
GTCGTCCTCATGGCTGAACCTGGAGAGGGTTTTCTGGGATTTCTTTTTCCAGGTATCCACCATGGTAAAGTCACTCATTTAATACAT
TTTACATGGCAAATATTTGAATATAACATACTATGAGTTGTCAATATATGTGGCCAAGTAGAAGCACTTTGATTCAATAATAA
TCAAAATAACAATCACTAAGCCATTAAATAATTGAATTAATTAATTTGTTTTTAAAAAATAATAGAATAACTGGATCTTTATGCTAA
AATAATTAACCTAAATTCAGATTTTACCACTCAAGATTGAACACTACTTAAAGTATGTAAAAAACAATCATCTGTATGTATAAT
TAAATACTAGTCCAGTTAATTGTTTTTATGGAATGTGTTAATTGACATATATCAATTGCTTGAACCTTATAATGTGCTTTGTTGTTTT

11

11

14

7

7

/

/

7

NRC-109
ATGAAGTTCCTCACTGCCACCTTCCTGGTGTTGTTTCATGGTCGTCCTCATGGCTGAACCTGGAGAGTGTTTTTTGGGATTGCTTTTTTCAC
GGGGTCCACCATGGTAGGGTCACGGAAGTAATTCGATTTTTACATGGCAAATATTTTAAAGATAAACACACCATATAGTAGTAGTCGATAT
ATTTGGCCAAATAGAAATCACTTTGATTTCAATAAATCAAAAATAACAATCTCTAGCGATTAAATATTTGCATTAATTTGGATTTTGT
TTTTAAAAATAGAAATAATCGGATCTTTATGGTAAATAATAAACAATACATCTCGATTTTACCAGTCAAGATTGAACACTACTTA
GAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTTAAACGAATAGTCCAAATAAATGTGTTATGTGAAATGTATTAAT
TGTCATTTTAATATCATTTTGCTTGAATTATCACCATTGTGTTTGTGTTTGTGTTTACACAGTTGAAAGTTGATCATCGGTGAAGGAC
TTCTACCATCACTTAGTGTATTTTTAATAGTATTATCATCGTAGTACTAATTATGGCAACACTTCTCTTGCTCGCTGACTCTCTCCATC

AGACTCATCCATGGCGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTTCGATGAA

//

NRC-110

GCCCACTTTGTATTTCGCAAGGTAAGAGCGATATATTTCAAATTCATTCCGATGAGACCAAGCATTGGGAAATGTGCTCAGCTTGTT
ACTGTTTAATGCAAAATGTTAACAATATCCTTTTTCTGTTGTTTTGTAGAAATGAAGTTTCGCTGCCGCTTCCTCATGATGTTTCATGG
TCGTCCTCATGGCTGAACCCGGAGAGGCTCGTTGGGGAACGTTCTTCAAACATATTTCAAAGGTAGAGTCACAGAATTAATTTGCT
TTTTACATTGCAAAATATTTTATATAACATAGCTGGAAAAATCAAAAAATAGGGCTTGATATATTTGGCAAAGTAGAATCCCTTTG
ATTTCAATAATAATCAAAATAAAAATCAGAAAGGCTTTGATTAGCATGTTCTTCAATAAAATGGACATTGTAGTTTATTTTGATT
CTCAAATGCACCAACCTGCTGCGGCAACAATTGAAATCAAATTTGTCTCCGAAACATTTAAAGTACATTTTCGAGGCAATTTAATC
TTTCTTTGTATCGAATTCGTTTTTAAAAATATAGAATAAATCTGGATCTTTATGCTAAAAATAATAAATCATACATTCTGATTTTACCAG
TCAAGATTGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTTTTAACTAATAGTCCTAATAATTG
TGTTATGGAATGTATTATTGTCATTTAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGCTGGAAG
GTTTCATCCATGGGTAAAGACTTCTACCATCATTTAGTGTATTTTAAATAGTATTATCATCAGTACTGTATTGATAACTTCTCTTG
TCTCGCTGACTCTCTCAGTCAGTCCAGGCACACAATGACGGCGAGCAGCAGGATCTCGACAAGCGCTCAGTGGATGATGAGC
CCAGTGTTATTGTTTTTGAATGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

NRC-111

GCCCACTTTGTATTTCGCAAGGTAAGAGCAATATATTTCAAATTCATTAGACGAGACCAAGCATTGGGATCTGTGCTCAACTTGTA
ACTGTATAATGCAAAATGTTAACAATATTTCTTTCTGTTGTTTTGTAGAAATGAAGTTTCGCTGCCGCTTCCTCATGATGTTTCATGG
TCGTCCTCATGGCTGAACCCGGAGAGGGTGCTTGGATGCTTGCCTTGAATAGGATCTATCATGGTAGAGTCACAGAATTAATTTGCT
TTTTACATTGCAAAATATTTTAAATATAACATGGCTGGAAAAATCAAAAAATGAGTACTCGATATATTTGGCAAAGTAGAATCCCTTTG
ATTTCAATAATAATCAAAAAACAATCAAAAAGGCCATTGATTAGCATGTTCTTCAATGAAATGGACATTGTAGTTTATTTTGATT
CTGACATGCACCAACTTGTCTGCGGCAACAATTGAATTCAAATTTGTCTCAGAAAAATTTAAAGTACATTTTCTTCCATTAGTCGG
ATTTGTTTTAAAAAATACAGAATAAATCTGGATCTTTATGCTAAAAATAATAAATCATACATTCTGATTTTACCAGTCAAGATTGAACGC
TACTTAAAGTATGTATAAAACATCATCTGTATTGATAATTGTTTAACTTTTAACTAATAGTCCTAATAATTGTGTTATGGAATGT
ATTCAATTGTCATTTAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGCTCTACTGAGGATCAATCGGT
AAGGACTTCTACCATCTAGTGTAAATTTAATAGTATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTTGTCTGGCTCTC
TCCATCAGCCAAATGGTGTATTATCGTGGCACTGGCAGCGTGACGTGAGCAGCAGGCTCTCGACAAGCGCTCAGTGGAGGACCAG
CCAGTTCTATTGCTTCTGCTTGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

NRC-112

GCCCACTTTGTATTTCGCAAGGTAAGAGCAATATATTTCAAATTCATTAGACGAGACCAAGCATTGGGATCTGTGCTCAACTTGTA
ACTGTATAATGCAAAATGTTAACAATATTTCTTCTCTGTTGTTTTGTAGAAATGAAGTTTCGCTGCCGCTTCCTCATGATGTTTCATGG
TCGTCCTCATGGCTGAACCCGGAGAGGGTGCTTGGATGCTTGCCTTGAATAGGATCTATCATGGTAGAGTCACAGAATTAATTTGCT
TTTTACATTGCAAAATATTTTAAATATAACATGGCTGGAAAAATCAAAAAATGAGTACTCGATATATTTGGCAAAGTAGAATCCCTTTG
ATTTCAATAATAATCAAAAAACAATCAAAAAGGCCATTGATTAGCATGTTCTTCAATGAAATGGACATTGTAGTTTATTTTGATT
CTGACATGCACCAACTTGTCTGCGGCAACAATTGAATTCAAATTTGTCTCAGAAAAATTTAAAGTACATTTTCTTCCATTAAATCGG
ATTTGTTTTAAAAAATACAGAATAAATCTGGATCTTTATGCTAAAAATAATAAATCATACATTCTGATTTTACCAGTCAAGATTGAACGC
TACTTAAAGTATGTATAAAACATCATCTGTATTGATAATTGTTTAACTTTTAACTAATAGTCCTAATAATTGTGTTATGGAATGT
ATTCATTGTCATTTAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGCTCTACTGAGGATCAATCGGT
AAGGACTTCTACCATCTAGTGTAAATTTAATAGTATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTTGTCTGACTCTC
TCCATCAGCCAAATGGTGTATTATCGTAGGCACTGGCAGCGTGACGTGAGCAGCAGGCTCTCGACAAGCGCTCAGTGGAGGACCAG
CCAGTTCTATTGCTTCTGCTTGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

NRC-113

ATGAAGTTCACTGCCACCTTCTGGTGTTGTTTCATGGTCGTCCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGTTCCT
AAAGGTGCCAAGCACCTTGGCCAGGCGGCCATTAAAGGTTTGGCCTCTTGCAGAGAGCAGCAAGAGCTCGACAAGCGCTCAGAGGAT
GACGAGCCCAAGTGCTATTGTTTTTGAA

//

NRC-114

ATGAAGTTCACTGCCACCTTCTGGTGTTGTTTCATGGTCGTCCTCATGGCTGGATCCGGAGAGTGTGGTTGGAAAAAGTGGCTCCGT
AAAGGTAGAGTCATGGATTTAATTTGCTTTTTACATTGCAAACTTTAATATAACATAGTTGGAAAAATCAAAAAATAGTAGTCG
ATATATTTGGCCATATAGAATCACTTGTATTCAATAATAATCAAAACAACATCAAAAAGGCCATTGATTAGCATGTTCTTCACT
AAAATGGACATTGTCTATTATTTTGATTCTCAGAGCACCACCTGCTGCGGCAACAATTTGAATCAAAATTTGTCTCAGAAATTC
AAAGTACATTGTTCTAGGCGATTTAATCTTTCCATTTCATCGGATTGTTTTTAAAAATATAGAATAAATCTGGATCTCTATGTTTAAAT
AATAAAACACACATTCTGATTTTACCTGTCAAGATTGAACAGCACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTT
AACTGTCAACTAATAGTCCAAATAATTGTTTATGGAATGTATTCAATTTGCTATATAATATCATTGCTTGAATTTATCACCATGTG
TTTTTTGTTGTTTTTACACAGGTGCCAAGCACCTTGGCCAGGCGGCCATTAAAGTAAAGACTTCTACCATCATTACTGTGTAAATTTA
ACAGTATTATCATCAGTACTGTTATTGACAACCTCTTGTCTCTGTGACTCTCTCCAGGGGTTTGGCCTTTCGCAAGAGCAGCAG
GAGCTCGACAAGCGCTCAATGGATGACGAGCCAGTGCTATTGTTTTTGAAGTGAAGAAGTCGCTTGAAGAGCCTTCAG

//

NRC-115

GCCCACTTTGTATTTCGCAAGGTAAGAGCGATATATTTCAAATTCATATAGACGAGACCAAGCATTGGGAAATGTGCTCAGCTTGTT
ACTGTATAATGCAAAATGTTAACAATATTTCTTCTGTTGTTTTGTAGAAATGAAGTTTCGCTGCCGCTTCCTGGTGTTGTTTCATGG
TCGTCCTCATGGCTGAACATGGAGAGGGTTTTGGGGATTTCTATATGAAGCCTGGTAGAGTCACGGAATTAATTCGATTTTAAACATG
GCAAAATATTTACTATAACATACCATATGAGTAGTCGATTAATTAATTTGATTGTTTTTAAAAATATAGAATAAATTTGGATCTTTAT
GCTAAAAATAATTAACATACATTTCTGATTTTACCAGTTAAGATTGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTT
AATTGTTTTAACTGTTAACTAATAGTCCAAATAATTGTTGTTGTTGGAATGTATTAATTTGTCATTTAATATCATTGCTTGAATTTGTC
ACCATGTGTTGTTGTTGTTTTTACACAGGTAGAAAGATTTCCATGGGTAAAGGACTTCTACCATCATTACTGTGTAAATTTA
TATTATCATCAGTACTGTTATTGATAACTTCTCTTGTCTGCTGACTCTCTACAGGTACATCAGAAGTCCTTATGGTTACACGAGC
AGCAGGAGGTGCAAGCGCTCAGTCGATGACAACCCAGTGCCATTGCTTCTGACTGAAGAAGTCGCTTGAAGGAGCCTTCAG

//

NRC-116

ATGAAGTTCACTGCCACCTTCTGGTGTTGTTTCATGGTCGTCCTCATGGCTGAACCTGGCGAGGGTTATTGGCGCTTCGCAACCAAC
CGTGGTGAAAGGTTATCCAGAGGCATTTCGCTGACGTGAGCAGCAGGAGCTCGACAAGCGCTCAGTGGATGACGAGCCAGTTCT
ATTGCTTTTTGA

//

NRC-117

ATGAAGTTCAC TGCCACCTT CCTCGTGTGTTTCATGTCATGTTTGAACCTGGAGAGTGT TTTTGGAA TGCTTTTTCACCGGGTCCA
CCATGGTTCGGGTCACGGAAGTAGTTCGATTTTACATGGCAAATATTTAAATGAAACATACCATATGAGTAGTCGATATATTTGGCC
AAGTAGAATCACTTTGACTTCAATAATAATCAAAAACATAATCAAAAAGCCCATTTGATTAGCATGTTCTTCAATGAAATGGACATT
GAGGTTTATTTTGATTCTCAGGCACCAACCTGCTGCGGCAACAATTGCATTCAAATTTGTCCCAAGAAACTTAATTAACATTTT
CTGGCGATTTAATCTTTGCATAAATTTGGATTTGTTTTTAAAAATATAGAATAACTGGATCTTTATGCTCAAATAATTAATCATACAT
TCTTATTTTATCAGTCAAGATTGAACGCTACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTTTTAACTAAA
AGTCCATAATAATTGTGTTATGGAATGTATTAATTGTCATTATAATCATTTCTTGAATTTATCACCATGTGTTTTGTTTGGTTTT
TTACACAGCTGGAAGGTTGATCCATAGGTAAGGACTTCTACCATCATTACTGTATAATGTTAATAATAGCATTATCATCAGTACTGT
TATTGATAACTTCTCTGTCTGCTGACTCTCTCCATCAGATTCAAAACGTCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGC
GCTCAGTGGATGACGAGCCAGTTCATTGCTTTTGCCTGAAGAAGTGCCTTG

//

NRC-118

ATGAAGTTCAC TGCCACCTT CCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGACT
GCGGTCCACAATGGTAAGTCAAGGAATTAATTGATTTTACGTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATATA
TTTGACCAAGTAGAATCACTTTGACTTCAATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAAATTGGATTGTT
TTTAAAAATATAGAATAACTGGATCTTAATGCTAAAAATAATTAACATACATTCTGATATTACAGTCAAGATTGAACGCTACTTAA
AAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTCGACTAATAGTCCATAAATTGTGTTATGGAATGTATTCAAT
GTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGCTGGAAGGTTGATCCATAGGTAAGGACT
TCTACCATCATTACTGTATAATTTAAGAGCATTATCATCAGTACTGTATTGATAACTTCTCTTGTCTCGCTGACTCTCTCCATCA
GACTACTCGGCTTTCATCATGGGCTTCCGGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGTGGATGAGG
AGCCAGTTCATTGCTTTTGAAGTGAAGAAGTGCCTTGAAGGAGCCTTCAG

//

NRC-119

ATGAAGTTCAC TGCCACCTT CCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGACT
GCGGTCCACAATGGTAAGTCAAGGAATTAATTGATTTTACTTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATATA
TTTGACCAAGTAGAATCACTTTGACTTCAATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAAATTGGATTGTT
TTTAAAAATATAGAATAACTGGATCTTAATGCTAAAAATAATTAACATACATTCTGATATTACAGTCAAGATTGAACGCTACTTAA
AAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTCGACTAATAGTCCATAAATTGTGTTATGGAATGTATTCAAT
GTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGTTGGAAGGTTGGTCCATGGGTAAGGACT
TCTACCATCATTACTGTATAATTTAAGAGCATTATCATCAGTACTGTATTGATAACTTCTCTTGTCTCGCTGACTCTCTCCATCA
GACTACTCGGCTTTCATCATGGGCTTCCGGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGTGGATGAGG
AGCCAGTTCATTGCTTTTGAAGTGAAGAAGTGCCTTGAAGGAGCCTTCAG

//

NRC-120

ATGAAGTTCAC TGCCACCTT CCTGGTGTGTTTCATGGTCTGCTCATGGCTGAACCTGGAGACTGTATTTTGGATTGATTGCGACT
GCGGTCCACAATGGTAAGTCAAGGAATTAATTGATTTTACTTGGCAAATATTTTAGTATAACATACCTTATGAGTAGTCGATATA
TTTGACCAAGTAGAATCACTTTGACTTCAATAATAATCAAAAATAACAATCTCTAGGCAATTTAATATTTGCATTAAATTGGATTGTT
TTTAAAAATATAGAATAACTGGATCTTAATGCTAAAAATAATTAACATACATTCTGATATTACAGTCAAGATTGAACGCTACTTAA
AAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTCGACTAATAGTCCATAAATTGTGTTATGGAATGTATTCAAT
GTCATATAATATCATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTACACAGTTGGAAGGTTGGTCCATGGGTAAGGACT
TCTACCATCATTACTGTATAATTTAAGAGCATTATCATCAGTACTGTATTGATAACTTCTCTTGTCTCGCTGACTCTCTCCATCA
GACTACTCGGCTTTCATCATGGGCTTCCAGGTTCTGGCACGGTGACGTCGAGCAGCAGGAGCTCGACAAGCGCTCAGTGGATGAGG
AGCCAGTTCATTGCTTTTGAAGTGAAGAAGTGCCTTGAAGGAGCCTTCAG

//

NRC-121

ATGAAGTTCAC TGCCACCTT CCTCGTGTGTTTCATGGTCTGCTCATGTTTGAACCTGGAGAGTGT TTTTGGAA TGCTTTTTCAC
GGGGTCCACCATGGTAGGGTCACAAAAGTGAATTTGATTATTACATGCCAAATATGTTAATGAAACATACCATATGAGCAGTCGTATT
ATTTGGACAAGTAGAATCACTTTGATTCTCAATAGTAATTAATAATAACAATCAAAAAGGCCCTTTGATTAGCATGTTCTTCAATGAAA
TGGACATTGAGGTTTATTTGATTCTCACCTGTCATCGACCTGCTGCGGCAACTATTGAAATCAAATTTGTCGAGAAAGAACTAAAT
TAACATTTTCTAGGCCATCTAATCTTTGTCATGAATTTGATTGCTTTTCAAAAATATAGAATAACTGGATATTTATGCTAAAAATAATA
AAAACACACATTCTGATTTTACCAGTCAAGATTGAACACTACTTAAAGTACGTATAAAACATCATCTGTATGTATAATTGTTTGC
TTTTAACAAATAGTCAAAATGATTGTTATGGAATGCAATTAATTGTCATTTAATATCATTTACTTGAATTTATCACCATGTGTTGT
TTGTTTTTACAGGTGGAGGTTTTCTCAATGCGCAAGGACTTACCATCATTACTGTGTAATTTTAAATAGTATTATCATCAGTAC
TCTTATTGACAACGCTCTCTGTCTGCTGACTCTCTCTATCAGATTAAACCCAGGGTATCGCGGTTACGACGAGCAGCAGGAGCTCG
ACAAGCGCGCAGTCGATGA

//

NRC-122

ATGAAGTTCAC TGCCACCTT CCTGGTGTGTTGCTTGGTCTGCTCATGGCTGAACCTGGAGAGGGTTTCTTTGGAGCCCTTCTCAAA
GGTAGAGTCACGGAATTAATTTGATTGTTACATGGCAAATATTTTGTATAACATATCATATGAGCAGTCGATGTTTGGACCAAGA
AGAATCATTTTGATTTCATAATAATAATCAAAAATAACAATCTCTGGAGATTATATTTGCAATAAATTGGATTTTATAAAATATAGAA
CAACTGGATCTTAATGCTAAAAATAATTAACATACATTCTGATTTTACCAGTCAAAATTAACCACTACTTTAAAGTATGTATAAAAC
ATCATCTGTATGTTAATTGTTTAACTTTTAAACAAATAGTCCAAATAATTGTGTAATGGAATGTAATTCATTGTCTATATAATATAGT
TTGCTTGACTTTATCACCGTGTGTTTTTGTGTTTTTTCACAGGTGCCAGGCTCCATGGGTAAAGGACTTACCATCATGACT
GTGTAAGTTTAAATAATATTATCATCAGTACTGTTTAAACGACTTCTCTTGTCTCGCTGACTCTCTCCATCAGAAATCATCCCAATG
CTCGTCACGGTTACGACGAGCAGCAGGAATCAACAAGCGCGCAGTCGATGA

//

NRC-123

GCCCACTTTGTATTTCGAAGGTAAGATCAATATTTTCAAATTCATTTAGACGAGACCAACCGTTTGGCAAATGTGCTCAGCTTGT
ATTGTATAATAACAAAGTTAACGATCTTTATTTTCTGTTTTTTGTAGAAATGAAGTTCACGCACTTCTGATGTTGTTTCATCT
TCGTCCTCATGGTTGAACCTGGAGAGTGTGTTGGAAAGATTGTTTTCTGAAGGCTAAGAAAGGTAGAATTCAGGAAATTAATTAGCT
TTTACATTGCAATAGATTTTTTATAACAGCTGGAATCAAAAATAAATAGTCGATATATTTGGCCAATTAGAATCACTTTAAT
TTCAATAATAATCTAAATAACAACCTAAAAGGCCCTTTGATTAGCATGTTCTTCAATGAAAAGGACATTGAGGTTTATTTGATTCT
CACATGCACCGACCTGTGCGGCAACAATTGAATTGAGTTTGTCCGAGAAATTCAAAGTACATTTTTCAGGCGATTAAATCTTT
CCATTACTCGGATTTAAATAATAAATAAGAAATCAAGCGCTATGATAAAATAATTACACATTCTGATTTTACAAGTCA
AGATTGAACACTATTAAAAGTGTGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTAATAGTCTTAATTAATTGTGTTATG
GAAATGTATTAAATTACATTTAATATCATTTGCTTGAGTTTACCATCATGTGTTTTGTTTGTGTTTTACACAGTTGGCAAGACTGTT

//

11

14

4

/

7

SUBSTITUTE SHEET (RULE 26)

AAGATTGAAAACCTTCTTAAAGGTACGTATAAAACATCATCTGTATTTATAATTGTTTAACTTTAAACAAATAATCCTACT
AATTGTGTTATGGAAATGTATAAAATGTAATTTAATATAATTTGCTTTAGTTTATCATTATTTGTTTTGTTTTGTTTTA
CACAGTTGGCAAGCATGTTGGCAAGGCGGCCCTTGAGTAAGAACTTCTACCATCATTACTGTATAATTTTGATAGTGTTA
TCACCAGTACTGTTATTGACAACCTTCTTGTCTGTGACTCTCTCCATCCGACTCATCCGAGTGCTTACCTCGGCGA
GAAGCAAGAACTCGACAAGCGCGCAGTCGATG

//

NRC-130

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGGGTTTTCGGATTGCTTTTTCAC
GGGATCCACCATGGTAGGGTCACGGAATTAATTAGATGTTTACATGGCAAATATTTTAAAGATAACACACCATATGAGTAGTCGATAT
ATTTGACCAATTAGAATCACTTTAATTTCAATAATAATCACAAATAACAATCTCTAGGCCATTTAATCTTTCCATTAAATCGGATTTGT
TTTTTTAAATATAGAATAAATGGATCTTTATGCTAAAATAATGAAACATACATTCTGATTTTACCAGTCAAGATTGAACGTTACTTA
AAAGTATGTTTTAAACATCATCTGTATGTATAATTGTTTAGCTGTAAACAAATAGTCCAAATAATTGTGTTATGGAAATGTATTAAT
TGTCATATAATATAATTTGCTTGAATTTATCACCATGTGTTTTGTTTGTGTTTTTAAACACAGCTGGAAAGTTGATCCATGGGTAAGG
ACTTCTACCATCATTACTGTGTATTTTAAATAGTATTATCATCAGTATGTTATTAACAACCTTCTCTTCTATCGCTGACTCTCTCCA
TCAGACTCATCCATCATGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

NRC-131

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGGGTTTGGGAAATTGGATGGGGCCC
CATATCAGCGGTAGAGTCACGGAATTAATTTGCTTTTTCCATTGCAAATATTTTAAATATTGCATAGCTGGAAATCAGGAAATAAGT
AGTCGATATATTTGGCCAAATAGAATAACTTTGATTTCAATAATAATCAAAATTACAATCAAAAAGGCCCTTGATTAGCATGTTCTCT
TCAATAAAATGGACATTTGAAGTTTATTTGATGCTCAATGCACCGACCTGCTGCGGCAACAATTGAAATCAAATTTGTCTCAGAAT
TTAAAGTACATTTTTCTAGGTGATTTAATCTTCCATTCACTGATTTATTTTATAAATATAGAATAAATGGATCTTTCTGCTAAAA
TAATAAAACACACATTTCTGATTTTACCAGTCAAGATTGAACACTACTTAAAGTATGTATAAAACATCATCTGTATGTATAATTGT
TAACGTGTTAACAATAGTCCAAATAATTGTGTTAAGGAAATGTATTAATTGTCTATTAAATATCATTTTGCTTGAATTTATCACCATGAG
TTTTTTGTTTTGTTTTTACACAGGTAGAAAGAAGGCCCTTGAGTAAGGACTTCTACCATCATTACTTTGTAATTTTTATAGTATTATC
ATCAGTACTGTTATTGACAACCTTCTCTTGTCTCGCTGACTCTCTCCATCAGGATGAACCTCAGAGCGTCGCAGTTACGACGAGTAGCA
GCAGAAGCTCGACAAGCGCGCAGTCGATGA

//

NRC-132

ATGAAGTTCACTGCCACCTTCTGGTGTGTTTCATGGTCGTCTCATGGCTGAACCTGGAGAGTGTTTTTGGGATTGCTTTTTCAC
GGGGTCCACCATGGTAGGGTCACGGAAGTAATTCGATTTTACATGGCAAATATTTTAAAGATAACACACCATATGAGTAGTCGATAT
ATTTGATATATTAGAATCACTTTGATTTCAATAATAATCAAAATAACAATCTCTAGGCCGATTAATATTTGCATTAATTGGATTTGT
TTTTAAAAATATAGAATAAATGGATCTTTATGGTAAAATAATTAAACATACATTCTGATTTTACCAGTCAAGATTGAACACTACTTA
GAAGTATGTATAAAACATCATCTGTATGTATAATTGTTTAACTGTTAACTAATAGTCCAAATAATTGTGTTATGGAAATGTATTAAT
TGTCATTTAATATCATTGCTTGAATTTATCACCATGTGTTTTTGTGTTTTTACACAGTTGGAAATTTGATCCATGGGTAAGGAC
TTCTACCATCATTACTGTGTATTTTAAATAGTATTATCATCAGTACTGTTATTGACAACCTTCTCTTGTCTCGCTGACTCTCTCCATC
AGACTCATCCATCAGGTTACGACGAGCAGCAGGAGCTCGACAAGCGCGCAGTCGATGA

//

NRC-133

GCCCCATTTGTATTTCGAAAGGTAATATCGATATTTTTCAAACCTCATTTAGACGAGACCAGGCATTTGGGAAACGTGCTAAGGTTGTTACT
ATGCAAAATTAATGATCTTTATTTTTCTGTTTTTTTTGTCAGAATGAAGTTCACTGCCACCTTCTCATGATTTTAAATCTTCTGCTCTCAT
AACCTGGAGAGTGTGGTATTAGGAAATGGTTTTAAAAGGCTGCTCACGGTAAAGTCACGGAATTAATTTGCTTTTTGCTTTACAAATATT
ACAGCAGCTGGAAATCAAAAAATAATAGTCGATGTTTGGCCAATTAGAATCACTTTGATTTCAATAATAATCTAAATAGCAACCT
GCCTTTGATTAGCATGTTCTTCAATGAAATGGGTGTTGAGGTTTTATTTTATTCTCATATGCACCGACCTGCTGCGGCAACAATTGAAT
TTTGTCCAAAGGAATTCAAAGTAACTTTCTAGGCGATTTAATCTTTCCATAACTCGGCTTTGTTTTTAAAAATATATAATAACTCAA
ATGATAAAATAATAACACATACATTCTGATTTATACAAGACAAGATTGAAAACCTTCTTGAAAGTATGTATCAAACATCATCTGTTTATAT
TTTAAACATTTCAAAAAAGTCCAACATAATTGTGTTATGGAATTGTATAAATTGTCAATTAATATAATTTTTTTGAGTTTATCAATATGTG
GTTTGTGTTTACACAGTTGGCAAGAAAGTTGGCAAGGTGGCCCTTAAGTAAGGACTTCTACCATTATTACTGTATAATTTTGATAGTATTA
AGTACTGTTATTGACAACCTTCTTTTTCTGCTGACTCTCTCCATCCGACTCATCTGCAAGTGCTTACCTTGGCGAGCAGCAGCAGCTCGA
GTGCAAGTCGATGAAGAGCCAGTGTTATTGCTTTTGAAGGAGTGCCTTGAAGGAGCCTTC

//

Appendix II. Nucleotide sequences of hepcidin-like genes and cDNAs referred to in Table 11.

NRC201

CGCCCTTAAGATGAAGACATTTCAGTGTTCAGTTGCAGTGGTGGTCGTCCTCGCATGTATGTTTCATCCTTGAAAGCACCGCTGTTCC
TTTCTCCGAGGTGCGAACCGGAGGAGTTGAAAGCATTGACAGTCCAGTTGGGGAACATCAACAGCCGGGCGGCACGTCCATGAATCT
GCCGGTACGTTCAATTTAGTGAATGAATTAAGTAATTACCTTAGCAAATTAACATCTAAGTGGTTGCGTTTACCCCTTGAATTGA
ATTAGCCCACTAGCGCTAGTTGTTAACCATTTGATTGTGAGCCGGTAGAGAGGGCTTCAGGGCGAGTAGTGTGAATACCTTGTGAAGT
GGAGACTTGGACAAAACTACTTACCATGTGCTTGTTCACCTTTTTCATTTTCTTTCTTGGCTGAGATACAGATGCATTTCAGGT
TCAAGCGTCAGAGCCACCTCTCCCTGTGCCGTTGGTGCTGCAACTGCTGTCAACAAGGGCTGTGGCTTCTGCTGCAATTCAGG
GACCTGCCAGCAAAGGGCGAATTCGTTTAAAAACAC

//

NRC202

AGATGAAGACATTTCAGTGTTCAGTTGCAGTGGTGGTCGTCCTCGCATGTATGTTTCATCCTTGAAAGCACCGCTGTTCCCTTCTCCG
AGGTGCGAACCGGAGGAGTTGAAAGCATTGACAGTCCAGTTGGGGAACATCAACAGCCGGGCGGCACGTCCATGAATCTGCCGATGC
ATTTTCAGGTTCAAGCGTCAGAGCCACCTCTCCCTGTGCCGTTGGTGCTGCAACTGCTGTCAACAAGGGCTGTGGCTTCTGCTGCA
AATTCGAGGACCTGCCAGCA

//

NRC203

ACGAGGTCCCTCATCCGCTGACACCAAAAGAACATCAATCAACTTTGGAAGTCTGCTTAGTGCAATGAAAATTTGTGCGTT
GGAGAGCGTCGCTTTTGGGAACATTGAAGAGTTCTGATCTTCCCTATAAACTGTCACTTCAATTTCAACTGATTTCAAC
AGGACTTTTAAATAGGCTATAAACTTCTTAAAAAAACGAGAATGAAGGCCTTTAGTGTTCAGTGGTACTCGTCAATTGC
ATGTATGTTTCATCCTTGAAAGCACCGCTGTTCCCTTCTCCGAGGTGCGAACCGGAGGAGTTGGAAGCTTTGACAGTCCAG
TTGGGGAACATCAACAGCCGGGCGGCAGTCCATGCATCTGCCGAGCCCTTTTCAGGTTCAAGCGTCAGATCCACCTCTCC
CTGTGCGGTTTGTGCTGCAACTGCTGTGCAACATTGGCTGTGGCTTCTGCTGCAAAATCTAAGGACCTGCCCGCAACAT
TTTCTAGTTTGTACATGTTTGAATGTTTCTTCTGAGATGTTGTTTTTGTGACTATGATAATGATTTATAAAATCACT
TCTTATTGTGACACTTTAAAAAAATAAACACATTCCTTTGAATCAAAAAAATAAAAAA

//

NRC204

CGAACCGGAGGAGGTTGAAAGCATTGACAGTCCAGTTGGGGAACATCAACAGCCGGGCGGCACGTCCATGAATCTGCCGAT
GCATTTTCAGGTTCAAAACGTGAGAGCCACCTCTCCCTGTGCCGTTGGTGCTGCAACTGCTGTGCAACAAGGGCTGTGGCT
TCTGCTGCAAAATCTGAGGACCTGCCAGCACTAAGCCATTTTATTAACTTATCGCCTTTAATTTGCCCTATTTCTTCTA
TGTTTCTTTTGGACTCTGTGGAGAAGATGCAATCTCATTGACGCTTTTATCACTGCACAACCTCAATCTTGT

//

NRC205

AAGATGAAGACATTTCAGTGTTCAGTGGTACCCGTCATTGTCATGTATGTTTCATCCTTGAAAGCACCGCTGTTCCCTTCTCCGAGGT
CGAACCGGAGGAGGTTGGAAGCTTTGACAGTCCAGTTGGGGAACATCAACAGCCGGGCGGCACGTCCATGAATCTGCCGATGCATTT
AGGTTCAAGCGTCAGAGCCACCTCTCCCTGTGCCGTTGGTGCTTCAACTGCTGTGCAACAAGGGCTGTGGCTTCTGCTGCAAAATC
TGAGGACCTGCCAGCA

//

NRC206

TAAGATGAAGCAATTCAGTGTGGCAGTGGTACTCGTCATGGCATGTATGTTTCATCGTGAAAGCACCGCTGTTCCCTTCTCCGAGGT
GCGAACCGGAGGAGGTTGGAAGCTTTGACAGTCCAGTTGGGGAACATCAACAGCCGGGCGGCAGTCCATGCATCTGCCGAGCCTTT
CAGGTTCAAGCGTCAGATCCACCTCTCCCTGTGCCGTTGGTGCTTCAACTGCTGTGCAACAATGGCTGTGGCTTCTGCTGCAAAAT
CTGAGACTGCCAGCA

//

NRC207

ACGAGGCACACGCTGACCAGGGGGTCAACCAAACTTCTGAAGAGACCCAGGTTCCCTAGAGAGCCACTAGAGAATCACCCG
GGAGCCCGAAGAACACAGGACGCTGCGGTGCTCGTCGTTGGCCGGACACCCATGAGACAGAAGACCTACAAGCCTCTCAG
CTTCAGAAGGATTTCTGACTCAGCATCTAAAACCTCCCTCAAATGAAGGCATTGACGATTGCAAGTGCAGTGCACCTC
GTGCTGCGCTTTGTTTGGCATTGAGTGCAGTCTGCGCTCCCTTCAAAGGGGTGACGAGCTGGAGAGGCGGGGGGCAA
TGACACTCCAGTTGCGGAACATCAAGTATGTCAATGGAATCCTGGATGGAGAATCCCACAGGCAGAAGCGCCACATCA
GCCACATCTCCCTGTGCCGCTGGTGCTGCAACTGCTGCAAGGCCAACAAAGGGCTGTGGCTTCTGCTGCAAGTTCTGAGGA
TTCCCGCAACACAACTCACAATGTATTAATTTATTACACTTTTGTGCGAGAAATGTCCTTTTCTTGACCTCTTTTGT
ATTTTGTATAATCTTTTAAATAAAACCGGGTACGATTTCATGAAAAAACCTTTGAATAAAATAAAAAAATAAAAAA
AAAAAC

//

NRC208

AAGATGAAGACATTTCAGTGTTCAGTTGCAGTGCACACTCGTCTCGCCTTTGTTTGCATTGAGGACAGCTCTGCCGTCCTTCCAG
GGGGTAAGAACGCAACTTTAACTCGCTTCATTTGCTTATTAGCCATAAATGTTTTGTCAGGATGCTGAGACACGGCTCTAAATGTG
TATAATTCATTAACAGGTGACAGGCTGGAGGAGGAGGGGCAATGACACTCCAGTTGCGGCACATCAAATGATGTCAATGGAATC
GTGGATGGTATGTTCAATCTGTTCAATCGACTGGATGAATTAAGCCAATTACTGTGAGCGCGTTAATTTAAGTGGCTGTGTTCCA
GCCCCGTGCTGTAGGGAATAAAACCCCTCGTTTCATGTGCTTGTCCGTCACAGGAGAGTCCCGTCAGGCAGAAGCGTCACATCAGC
CACATCTCCATGTGCCGCTGGTGCTGCAACTGCTGCAAGGCCAAGGGCTGTGGCCCTGCTGCAAAATCTGAGGACCTGCCAGCA

//

NRC209

AAGATGAAGACATTTCAGTGTTCAGTGCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGGCACCTTCTCTGAG
GTAAGCTCCTGACTTCAGATCGTTTCATTTGCTTGTATCCATGAATCTCTCATCAACAGACTGAGACTTGATTCTTCTTTATCA
GGTACAAGAGCTGGAGGAGGAGTGAAGCAATGACAAATGACAGTGTGAACATCAGGAGACATCAGTGGACTCATGGATGGTAGGTTCA
AGTTCACTGAATGGATCAAACCAATTCACATCAGACCTTTTCAGATGGAAGTGAATGTGTTTTAGTCTCAAAGGTGCCCTGAAGCTCA
GTTTACACAAGCAGTGAAAAACAAACACAGAAAGTTATGATGCTGATGAACCTTCTCTCATGTCTCATGTCTCTCACACAGATGC
CATACAACAGACAGAAGCGTGCTTCAAGTGAAGTTCTGCTGCGGCTGCTGCAAGAGCTGGTGTCTGTGGACTGTGCTGCAAGTTCT
GAGGATTCCTGCTCCAACAC

//

NRC210

ACGAGCTGACAGGAGCTGACAGGAGTCAACAGCAGAGTCAAAGAACTAAACAACCTTAACCTCAGTCAAACCTCTCAAAGATG
AAGACATTTCAGTGTTCAGTGCACAGTGGCCGTCGTCTCTGCTTTATTTGTATCCAGCAGAGCTCTGCCCTCTTCTCTGA

GGCACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAGCATCAGGAGACACCAGTGGACTCGTGGATGA
TGCCATACAACAGACAGAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGC
TGCAAGTTCTGAGGATTCCTGCTCCAACAACCATCAATATTCATTGTTTGCCTTTTGTCTTAAAGTTCAATTGAACATA
TAAACATATTTCTGGTTGAGCATGTGATAGTTTAAATGGTGTACTATTGGTTTCATGGTATAGTCAAGTGTTCAGAGATG
TGATTGTATCACCCACATATTTCTCTGTTAGGTGTATTTCAATAAATGCCAATGATCCTTTGAAAAA
AAAAA

//

NRC211

ACGAGCGGCACGAGGTGAACCTGACAGGAGCTGACAGGAGTCAACAGCAGAGTCAAAGAACTAAACAACTTAACCTCAGTCA
AACTCTCAAAGATGAAGACATTAGTGTGTCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTG
GCCTCCTTTCTGAGGCACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACACCAGT
TGACTCGTGGATGATGCCAAACAACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTG
TCTGTGGAATGTGCTGCAAGTTCTGAGGATTCCTGCTCCAACAACCATCAATATTCATTGTTTGCCTTTTGTCTTAAAGTTTAA
AGTTCAATTGAACATATATACATATTTCTGGTAGAGCATGTGATAGTTAATGGTGTACTCCTTGGTTTCATGGTGTAGTTA
AGTGTTCAGAGATGTGATTGTATCACCCACATATTTCTCTGTTAAGGTGTATTTCAATAAATGTTAATGCTCCTTTGAA
AAAAA

//

NRC212

ACGAGACTGACAGGAGCTGACAGGAGTCAACAGCAGAGTCAAAGAACTAAACAACTTAACCTCAGTCAAACCTCTCAAAGAT
GAAGACATTAGTGTGTCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTG
AGATGCCATACAACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCGGAGCTGGTGTCTGTGGAATG
TGCTGCAAGTTCTGAGGATTCCTGCTCCAACAACCATCAATATTCATTGTTTGCCTTTTGTCTTAAAGTTTCAATTGAA
CTATAACATATTTCTGGTTGAGCATGTGATAGTTAATGGTGTACTCATTGGTTTCATGGTATAGTCAAGTGTTCAGAG
ATGTGATTGTATCACCCACATATTTCTCTGTTAGGTGTATTTCAATAAATGCCAATGATCCTTTGAAAAA

//

NRC213

AAGATGAAGACATTAGTGTGTCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCTCCTT
TCCTGAGGTAAAGCACCTGACTTCAGATCGTTTCATTGCTTGTATCCATGAATCTCTCATCATCACTAGACTTGAT
TCCTTCTTTATCAGGCACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAGCATCAGGAGACACCAGTG
GACTCCAGGAGTGAATGTGTTTGTAGTCAAAAAGTGCCCTGAAGCTCAGTTTACACAAGCAGAGAAAACAAACAGAGTAA
GTTATGATGATGCTGATGAAGGTCTCCTCATGTCTCATGTCTCTCACACAGATTCCATACACAGACAGAAGCGTAGCTT
TAAGTGAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAGTTCTGAGGATTCCTGCTCCAAC
AAC

//

NRC214

AGATGAAGACATGTCAGTGTGTCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCTCCTT
CCTGAGGTAAAGCACCTGACTTCAGATCGTTTCATTGCTTGTATCCATGAATCTCTCATCATCACTAGACTTGAT
CCTTCTTTATCAGGTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACACCAGTTG
ACTCGTGGATGGTAGGTTAGTTCACTGAATGGATCAATCCATTTACATCAGATCTTTAGATGGAAGTGAATGTGTT
TAGTCAAAAAGTGCCCTGAAGCTCAGTTTACACAAGCAGAGAAAACAAACAGAGTAAAGTTATGATGATGCTGATGAAG
GTCTCCTCATGTCTCATGTCTCTCACACAGATGCCAAACAACAGACAGAAGCGTGGCTTTAAGTGAAGTTCTGCTGCGG
CTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAGTTCTGAGGATTCCTGCTCCGGACAA

//

NRC215

AAGATGAAGACAATCAGTGTGTCAGTCAAGTGGCCGTCGTCTCTCTTTATTTGTATCCAGCAGAGCTCTGCCTCCTT
TCCTGAGGTAAAGCACCTGACTTCAGATCGTTTAAATTTGCTTGTATCCATGAATCTCTCATCACTACTGAGACTTGAT
TCCTTCTTTATCAGGCACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACACCAGTG
GACTCAGGGATGGTAGGTTAGTTCACTGAATGGATCAATCCATTTACATCAGATCTTTAGATTGAAGTGAATGTGTT
TTAGTCAAAAAGTGCCCTGAAGCTCAGTTTACACAAGCAGAGAAAACAAACAGAGTAAAGTTATGATGATGCTGATGAAG
GTCTCCTCATGTCTCATGTCTCTCACACAGATTCCATACACAGACAGAAGCGTAGCTTTAAGTGAAGTTCTGCTGCGG
CTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAAATCTGAGGACCTGCCAGCA

//

NRC216

AAGATGAAGACATTAGTGGTGCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCTCCTT
TCCTGAGGTAAAGCACCTGACTTCAGATCGTTTCATTGCTTGTATCCATGAATCTCTCATCATCACTAGACTTGAT
TCCTTCTTTATCAGGTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACACCAGTT
GACTCGTGGATGGTAGGTTAGTTCACTGAATGGATCAATCCATTTACATCAGATCTTTAGATTGAAGTGAATGTGTT
TTAGTCAAAAAGTGCCCTGAAGCTCAGTTTACACAAGCAGAGAAAACAAACAGAGTAAAGTTATGATGATGCTGATGAAG
GTCTCCTCATGTCTCATGTCTCTCACACAGATGCCAAACAACAGACAGAAGCGTGGCTTTAAGTGAAGTTCTGCTGCGG
CTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAAATCTGAGGACCTGCCAGCA

//

NRC217

AAGATGAAGACATCAGTGGTGCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTGAG
GTAAGCACCTGACTTCAGATAGCTTCATTGCTTGTATCCATGAATCTCTCATCACTACTGAGACTTTATTCCTTCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGCGCATCAGGAGACATCAGTGGACTCGTGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGCGAATGTGTTTGTAGTCAAAAAGTGACCTGATGCTCAG
TTTACACAAGCAGAGAAAACAAAGCAGAGTAAAGTTATGATGATGCTGATGAACGTGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACCGAAGCGTAGCTTTAAGTGAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAAATCTG
AGGATTCCTGCTCCAACAAC

//

NRC218

AAGATGAAGACATTAGTGTGGCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTGAG
GTAAGCACCTGACTTCAGATAGCTTCATTGCTTGTATCCATGAATCTCTCATCACTACTGAGACTTGATTTCTTCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGCGCATCAGGAGACATCAGTGGACTCGTGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGCGAATGTGTTTGTAGTCAAGAAGTGCCCTGATGCTCAG
TTTACACAAGCAGAGAAAACAAAGCAGAGTAAAGTTATGATGATGCTGATGAACGTGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACCGAAGCGTAGCTTTAAGTGAAGTTCTGCTGCGGCTGCTGTAGAGCTGGTGTCTGTGGACTGTGCTGCAAAATCTG

AGGATTCTGCTCCAACAAC

//

NRC219

AAGATGAAGACATTTCGTGGTTGCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATAGCTTTCATTTGCTTGTATCCATGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGACATCAGGAGACATCAGTGGACTCGTGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGACTGTGTTTTAGTCACAAAAGTGCCCTGATGCTCAG
TTTACACAAGCAGAGAAAAACAAGCAGAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACAGAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAATTCTG
AGGATTCTGCTCCAACAAC

//

NRC220

AAGATGAAGACATCAGTGGTTGCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATAGCTTTCATTTGCTTGTATCCATGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGACATCAGGAGACATCAGTGGACTCGTGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGACTGTGTTTTAGTCACAAAAGTGCCCTGATGCTCAGT
TTACACAAGCAGAGAAAAACAAGCAGAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCCA
TACAACAGACATAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAATTCTGA
GGATTCTGCT

//

NRC221

AAGATAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAGG
TAAGCACCTGACTTCAGATCGTTTTTCATTTGCTTGTAGCCTTGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAGG
TACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCAGTGGACTTGTGGATGGTAGGTTCA
TTCAGTGAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGAAGCTCAGT
TTACACGAGCAGAGAAAAACAACACAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCCA
TACAACAGACAGAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGCCCTGGTGTCTGTGGACTTTGCTGCAGATTCTGA
GGATTCTGCTCCAACAAC

//

NRC222

AAGATGAAGACATTTCAGTGTTCAGTCGAGTGGCCGTCGTGCTCATCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATAGTTTCATTTGCTTGTATCCATGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCATTGGACTCATGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGATGCTCAG
TTTACACAAGCAGAGAAAAACAAGCAGAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAATTCTG
AGGACCTGCCAGCA

//

NRC223

AAGATGAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATAGTTTCATTTGCTTGTATCCATGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCATTGGACTCATGGATGGTAGGTTCA
GTTCACTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGATGCTCAG
TTTACACAAGCAGAGAAAAACAAGCAGAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACATAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAATTCTG
AGGACCTGCCAGCA

//

NRC224

AGATGAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAGG
TAAGCACCTGACTTCAGATAGTTTCATTTGCTTGTATCCATGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAGG
TACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCCGCTGAACATCAGGAGACATCAGTGGACTCGTGGATGGTAGGTTCA
TTCAGTCAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGATGCTCAGT
TTACACAAGCAGAGAAAAACAAGCAGAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
TACAACAGACCGAAGCGTAGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGAGCTGGTGTCTGTGGACTGTGCTGCAAATTCTGA
GGACCTGCCAGCA

//

NRC225

AAGATGAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCATCTTTATTTGTATCCAGCAGAGCTCTGCCACCTCTCTCTGAG
GTACAAGGGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCAGTGGACTCGTGGATGATGCCATAC
AACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGGCCCTGGTGTCTGTGGACTTTGCTGCAGATCCTGAGGA
TTCCTGCTCCAACAAC

//

NRC226

AAGATGAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATCGTTTCATTTGCTTGTAGCCTTGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCAGTGGACTTGTGGATGGTAGGTTCA
GTTCACTGAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGAAGCTCAG
TTTACACGAGCAGAGAAAAACAACACAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGACCTGGTGTCTGTGGACTTTGCTGCAGATTCTG
AGGATTCTGCTCCAACAAC

//

NRC227

AAGATGAAGACATTTCAGTGTTCAGTCACAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCTCTGAG
GTAAGCACCTGACTTCAGATCGTTTCATTTGCTTGTAGCCTTGAATCTCTCATCAACATACTGAGACTTGTATTCCTTTATCAG
GTACAAGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCAGTGGACTTGTGGATGGTAGGTTCA
GTTCACTGAATGGATCAAACCAATTACATCAGATCTTTAGATGGAAGTGAAATGTGTTTTAGTCACAAAAGTGCCCTGAAGCTCAG
TTTACACGAGCAGAGAAAAACAACACAGTAAGTTATGATGATGCTGATGAACGTCCTCATGTCTCATGTCTCTCACACAGATGCC
ATACAACAGACAGAAGCGTGGCTTTAAGTGTAAGTTCTGCTGCGGCTGCTGCAGACCTGGTGTCTGTGGACTTTGCTGCAGATTCTG
AGGATTCTGCTCCAACAAC

11

11

14

14

1

/

1

7

7

ACGAGCTGACAGGAGCTGACAGGAGTCACCAGCAGAGCTCAAAGAACTAAACAACCTTAAGTCTCAGTCAAAGTCTCAAAGATGAAGACAT
TCAGTGTGTCAGTCAAGTGGCCGTCGTGCTCGTCTTTATTTGATCCAGCAGAGCTCTGCCACCTTTCCTGAGGTACAGAGCTGG
AGGAGGCAGTGCAGCAATGACAATGCAGCTGTCAGCATCAGGAGACACCGAGTGGACTCAGGAGTGTGCCAAACAACAGACAGAAAGC
GCAGCGCCGATTTGTTGCCATGTTGCAATCAAATGGCTGTGGAAGTCTGCTGCAAGGTCTAAACAGACTCTTGGGCAGATCAATCCA
GGTTCGTCTTTGTTGTCCTCCGTGGAGTGCAGAACAGAGACCTTCTCAGCCCATAGTCCAAGTTTCTGCCACTAGACCACCGCCTC
TCCCTCATCAAATACCAATGTTTTTCATTTGTCTTAAAGTTCATTGAACTATAAACATATTTCTGGTAGAGCATGTGATAGTTTA
ATGGTGTACTCATTTGGTTCATGTGATAGTCAGATGTTCAGAGATGTGATTATATCATCCACATATTTCTCTGTTAAGGTGTACTG
TCAATAAATGTCGAATGCTCCTTTGAAAAAATAAAAAAAAAA

//

NRC237

CGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCCTGAGGTGAGCTCCTGACTTCAGATCGTTTCATTTAGCTTGTT
ATCCATGAATCTCTCATCAACATACTGAGACTTGAATCCTTCTTTATCAGGTACAGGAGCTGGAGGAGGCAGTGAGCAATGACAATG
CAGCTGCTGAACATCAGGAGACATCAGTGGACTCATGGATGGTATGTTTCACTTCACTGAATGGATCAAACCAATTACATCAGATCT
TTCAGATGGAAGTGAATTTGTTTTAGTCCCAAAGTGCCCTGAAGCTCAGTTTACACAAGCAGAGAAAAACAAAACACAGTAAGTTA
TGATGATGCTGATGAACGTCTCCTCATGTCTCATGTCTCTCACACAGATGCCATAACAACAGACAGAAGCGCAGCGCCGAGTGTAGCT
TCTGCTGCAATGAATCTGGCTGTGGAATTTGCTGCAAATTCCTGAGGATTCTGCTCCAACAACAAGGGCGAATTC

//

NRC238

AAGATGAAGACATTTCAGTGTTCAGTGCAGTGCAGTGGCCGTCGTGCTCGTCTTTATTTGTATCCAGCAGAGCTCTGCCACCTTTCCTGAG
GTGAGCTCCTGACTTCAGATCGTTTCATTTAGCTTGTTATCCATGAATCTCTCATCAACATACTGAGACTTGAATCCTTCTTTATCA
GGTACAGGAGCTGGAGGAGGCAGTGAGCAATGACAATGCAGCTGCTGAACATCAGGAGACATCAGTGGACTCATGGATGGTATGTTT
AGTTCACTGAATGGATCAAACCAATTCACATCAGATCTTTAGATGGAAGTGAATTTGTTTTAGTCCCAAAGTGCCCTGAAGCTCA
GTTTACACAAGCAGAGAAAAACAAAACACAGTAAGTTATGATGATGCTGATGAACGTCTCCTCATGTCTCATGTCTCTCACACAGAT
GCCATAACAACAGACAGAAGCGCAGCGCCGAGTGTAGCTTCTGCTGCAATGAATCTGGCTGTGGAATTTGCTGCAAATTCCTGAGGACC
TGCCAGCA

//

NRC239

GTGGAGGAGCCAGTGAGCAGTGAGAATGGAGCAAATGAACACACATAAGATCTTTCGGATGGAAGTGTATGTGTTTTAGTCACATGA
GTGGCTCGAAGCTCAGTACACACGAGCAGAGAGAACGAACACAGTGTGTTTTATTCTGCTTGTGTAAACTGAGCTTCAGTTTACACA
AGCAGAGAAAAACAAACACAGTAAGTTATGATGATGCTGATGAACGTCTCCTCATGTCTCATATCTCTCACACAGATGCCAAACA
GACAGAAGCGTGGCTCTAATTGCAAACCATGCTGCAATCATAATGGCTGTGGAACGTGCTGCGAAGTCTGAGGATTCTGCTCCACA

//